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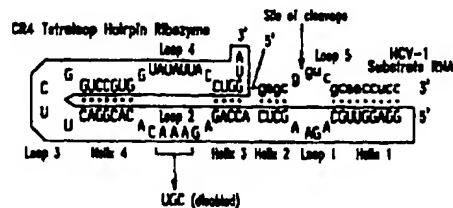
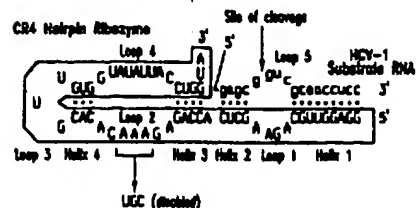
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(57) Abstract

This invention provides ribozymes useful to treat or prevent Hepatitis C Virus ("HCV") infection or disease in an organism or subject, as well as methods of treating an HCV infection or disease. Reagents such as vectors, host cells, DNA molecules coding for these ribozymes useful in methods of treatment and prevention of HCV infection or disease are also provided.



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Description

HEPATITIS C VIRUS RIBOZYMES

5 Technical Field

The present invention relates generally to ribozymes, and more specifically, to ribozymes which are capable of cleaving Hepatitis C Virus nucleic acids, as well as to methods which utilize such ribozymes.

10 Background of the Invention

Hepatitis C Virus ("HCV") is an RNA virus which is responsible for approximately 75% of all cases of non-A, non-B hepatitis. Based upon epidemiologic and serologic surveys, it has been estimated that at least 1% to 2% of the world population is chronically infected with HCV (Davis et al., "Therapy for Chronic
15 Hepatitis C" in *Gastroenterology Clinics of North America*, pp. 603-613, 1995). In the United States, approximately 150,000 acute cases occur annually, where it is the ninth leading cause of death. Moreover, approximately 50% of the acutely infected individuals go on to develop chronic liver disease, and of these, 25% will develop cirrhosis. In addition, on a worldwide basis, 50% of the cases of hepatocellular
20 carcinoma are correlated with HCV infection.

HCV is a positive-stranded RNA virus that is related to the flaviviridae family. The virus, which was isolated and characterized in 1989 (Choo et al., *Science* 244:362-364, 1989) has an ~9.5 Kb linear genome that replicates through a double stranded RNA intermediate, mediated by a virally encoded RNA-dependent RNA
25 polymerase. There is no known DNA intermediate in the replication process. Presumably, because its replication is exclusively through RNA polymerases, which lack proofreading activities, variability in the coding sequences is a hallmark of individual HCV isolates.

At present, the only therapy which shows some promise for the treatment
30 acute and chronic cases of HCV is alpha interferon (Fried and Hoofnagle, *Seminars in Liver Disease* 15(1):82-91, 1995). Treatment with alpha interferon however, particularly for chronic patients, produces only temporary results. In particular, in most studies where patients with chronic HCV infections are treated with alpha interferon, only 20% to 25% maintain a sustained, long-term response (Fried and Hoofnagle,
35 *supra*). In addition, treatment with alpha interferon can produce a wide array of side effects, including systemic effects (e.g., fatigue, fever, headache, anorexia, weight loss,

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nausea, vomiting, diarrhea, and hair loss), neurologic and psychological effects, an increased susceptibility to infections, as well as an assortment of autoimmune diseases.

The present invention provides an effective treatment to combat HCV infection, and further provides other, related advantages.

5

Summary of the Invention

The present invention provides ribozymes useful to treat or prevent Hepatitis C Virus ("HCV") infection or disease in an organism or subject, as well as methods of treating HCV infection or disease. Reagents such as vectors, host cells,
10 DNA molecules coding for these ribozymes useful in methods of treatment and prevention of HCV infection or disease also are provided.

Accordingly, in one aspect the present invention ribozymes are provided which have the ability to inhibit replication, infectivity, or gene expression of a hepatitis C virus. Within certain embodiments, the ribozyme is a hammerhead or hairpin
15 ribozyme. Within other embodiments, the ribozyme cleaves genomic strand RNA (either the positive or negative strand), representative examples of which include the sequences set forth in Table I, below. In other aspects, the present invention also provides nucleic acid molecules encoding such ribozymes where, within certain
20 embodiments, the nucleic acid molecule is DNA or cDNA. Within preferred embodiments, the nucleic acid molecule is under the control of a promoter to transcribe the nucleic acid molecule.

In another aspect, the present invention provides host cells containing the ribozymes described herein, vectors comprising a promoter operatively linked to the nucleic acid molecule which encodes the ribozymes described herein, and host cells
25 containing such vectors. Within certain embodiments, the vector is a plasmid, a viral vector, retrotransposon, or a cosmid. Representative examples of promoters include the polIII and CMV promoters.

In a further aspect, the present invention provides methods for producing a ribozyme, the ribozyme being able to inhibit hepatitis C viral infection and replication
30 in a cell, comprising the step of providing a nucleic acid molecule (e.g., DNA) encoding a ribozyme under the transcriptional control of a promoter (e.g., in a vector), and transcribing the nucleic acid molecule to produce the ribozyme. The method may also further comprise purifying the ribozyme so produced. The ribozyme may be produced *in vitro*, *in vivo* or *ex vivo*.

35 In yet another aspect, the present invention provides methods of interfering with or preventing HCV replication in a cell infected with HCV, comprising

the step of introducing into a cell an effective amount of the ribozymes described herein. In one embodiment, such methods comprise introducing into the cell an effective amount of DNA encoding a ribozyme as described herein and transcribing the DNA to produce the ribozyme.

5 In still a further aspect, the present invention provides methods of preventing hepatitis C viral infection in a cell susceptible to infection with HCV, comprising the step of introducing into the cell an effective amount of a nucleic acid molecule (*e.g.*, DNA) encoding a ribozyme as described herein and transcribing the DNA to produce the ribozyme.

10 In preferred embodiments, the methods further comprise administering the cell transduced with a retroviral vector to a mammal of the same species as that from which the transduced cell was obtained. In other preferred embodiments, the cell transduced with the retroviral vector has been obtained from the mammal receiving the transduced cell.

15 The above-described methods, as well as the compositions described herein, may be utilized to treat or prevent HCV infection or disease in a wide variety of warm-blooded animals or mammals, including for example, humans.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition,
20 various references are set forth herein that describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

Brief Description of the Drawings

25 Figure 1 is a schematic illustration of vector pGem7Z (Promega, Madison, WI).

Figure 2 is a schematic illustration of vector pLNT-Rz.

Figure 3 is a schematic illustration of one representative hairpin ribozyme (Seq. ID Nos. 70 and 71) and one tetraloop hairpin ribozyme (CR4) (Seq. ID
30 Nos. 73 and 71).

Figure 4 is an *in vitro* cleavage timecourse of CR2 ribozyme cleaving a short 5'UTR substrate.

Figure 5 is a graph which depicts *in vitro* cleavage timecourse reactions using CR2 and various facilitator RNA molecules, cleaving the long 5'UTR substrate.

Figure 6 depicts two *in vitro* cleavage experiments, one with and one without a pre-heating step of 90°C for 1 minute prior to the cleavage reaction. Ribozymes tested are CR2, CR6, CR7 and CR8 using the short 5'UTR substrate.

Figure 7 shows *in vitro* cleavage reactions using variants of CR4 ribozyme with either 8, 7 or 6 nucleotides in Helix 1, using the short capsid substrate.

Figure 8 depicts *in vitro* cleavage reactions using variants of CR4 ribozyme with either 8, 7 or 6 nucleotides in Helix 1 using the long capsid substrate.

Figures 9A, 9B, 9C and 9D are schematic illustrations of certain vectors described herein: pPur, pPur-HCV, pLNL6 and pLNL-Pur-HCV.

Figure 10 is an RNase protection assay verifying ribozyme expression after transfection.

Figures 11A, 11B and 11C are three graphs which show ribozyme cleavage obtained in co-transfection experiments.

Figure 12 is a schematic illustration of the experimental design used to test the protective effect of the ribozymes.

Figure 13 is a graph showing ribozyme cleavage and subsequent protection of cells from incoming HCV-containing positive-stranded RNA virus.

Figure 14 is a western blot indicating detection of HCV nucleocapsid protein.

Figure 15 is a schematic illustration of vector pGL3 (Promega, Madison, WI).

Figure 16 is a schematic illustration of adenoviral vectors expressing either β -galactosidase or ribozyme.

Figure 17 is a graph which shows FPLC data generated during purification of adenoviral vectors from cell lysates.

Figure 18 is a schematic illustration of an AAV ribozyme expression vector.

Figure 19 is an autorad which shows the results of *in vitro* cleavage of short (<50 nucleotides) substrates by two ribozymes (CNR3 and CNR6) against the (-) strand of HCV.

Figure 20 is an autorad which shows the results of *in vitro* cleavage of long substrates by two ribozymes (CNR3 and CNR6) against the (-) strand of HCV.

Figure 21 is a schematic illustration of vectors pLNT-Rz, pAvC-Rz, pAvM-Rz, pAvCM-Rz and pAAVM-Rz.

Figure 22 is a schematic illustration of pAMFT.dBAM.

Figure 23 is a schematic illustration of vectors pPur-HCV, pLNL-PUR-HCV and pGem4-HCV 5'C.

Figure 24 is a western blot which shows expression of HCV core protein following transfection into HT1080 cells.

5 Figure 25A is a blot which shows the ability of different promoter/vector combinations to reduce viral core expression in cell culture. pAAVM-CR4 shows the greatest efficacy. Figure 25B is a blot which shows the further titration of pAAVM-CR4 and its effect on viral core expression.

10 Figure 26 shows the results of an RNase protection assay performed to measure expression of ribozyme CR4 in human liver cells.

Figure 27 is a table which compares the infectivity of primary normal human hepatocytes with either recombinant adenovirus or recombinant adeno-associated virus carrying the β -galactosidase gene.

15 Detailed Description of the Invention

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

20 "Ribozyme" refers to a nucleic acid molecule which is capable of cleaving a specific nucleic acid sequence. Ribozymes may be composed of RNA, DNA, nucleic acid analogues (e.g., phosphorothioates), or any combination of these (e.g., DNA/RNA chimerics). Within particularly preferred embodiments, a ribozyme should be understood to refer to RNA molecules that contain anti-sense sequences for specific recognition, and an RNA-cleaving enzymatic activity.

25 "Ribozyme gene" refers to a nucleic acid molecule (e.g., DNA) consisting of the ribozyme sequence which, when transcribed into RNA, will yield the ribozyme.

30 "Vector" refers to an assembly which is capable of expressing a ribozyme of interest. The vector may be composed of either deoxyribonucleic acids ("DNA") or ribonucleic acids ("RNA"). Optionally, the vector may include a polyadenylation sequence, one or more restriction sites, as well as one or more selectable markers such as neomycin phosphotransferase, hygromycin phosphotransferase or puromycin-N-acetyl-transferase. Additionally, depending on the host cell chosen and the vector employed, other genetic elements such as an origin of
35 replication, additional nucleic acid restriction sites, enhancers, sequences conferring

inducibility of transcription, and selectable markers, may also be incorporated into the vectors described herein.

As noted above, the present invention provides ribozymes which are
5 capable of cleaving Hepatitis C Virus nucleic acids. Briefly, the viral genome of HCV
has an approximately 9.5 Kb linear genome that replicates through a double stranded
RNA intermediate (Choo et al., *Proc. Natl. Acad. Sci. USA* 88:2451-2455, 1991; Choo
et al., *Brit. Med. Bull.* 46(2):423-441, 1990; Okamoto et al., *J. Gen. Vir.* 72:2697-2704,
1991; see also, e.g., Genbank Accession No. M67463, Intelligenetics (Mountain View,
10 California). This sequence expresses a polyprotein precursor of 3011 amino acids,
which is cleaved to yield several different viral proteins, including C (nucleocapsid
protein) E1, E2/NS1, and non-structural proteins NS2, NS3, NS4, and NS5 (Houghton
et al., *Hepatology* 14:381-388, 1991).

Sequence analyses of HCV isolates indicate considerable sequence
15 variation (for reviews see Houghton et al., *Hepatology* 14:381-388, 1991; van Doorn, *J.
Med. Virology* 43:345-356, 1994; Bukh et al., *Seminars in Liver Disease* 15:41-63,
1995; Simmonds, *Hepatology* 21:570-583, 1995; see also Genbank Assession Nos.
D10749, D10750, D11168, D11355, D13558, D30613, D90208, L02836, M58335,
M84754, M96362, S62220, U01214, U16362, X61596). The most conserved regions
20 (>90% sequence identity within the known isolates) reside within the first 1000
nucleotides of the genome, which consists of a 5' untranslated region (5'UTR) and the
coding region for the nucleocapsid (Bukh et al., *Proc. Natl. Acad. Sci. USA* 89:4942-
4946, 1992). The highest degree of sequence variability (up to 50% sequence
divergence) is found within the E1 and NS2 genes (see above reviews). Based on this
25 sequence heterogeneity, HCV isolates can be categorized into at least 12 distinct
genotypes (Okamoto et al., *Virology* 188:331-341, 1992; Bukh, et al., *Proc. Natl. Acad.
Sci. USA* 90:8234-8238, 1993). Although most of the HCV genotypes are distributed
worldwide, certain strains are found in discrete geographical regions (Bukh, et al., *Proc.
Natl. Acad. Sci. USA* 90:8234-8238, 1993) and their distribution may play a role in
30 ribozyme-mediated therapy.

RIBOZYMES

As noted above, the present invention provides ribozymes having the
ability to inhibit replication, infectivity, or gene expression of a hepatitis C viral
35 infection in a cell. Several different types of ribozymes may be constructed for use
within the present invention, including for example, hammerhead ribozymes (Rossi, J.J.

et al., *Pharmac. Ther.* 50:245-254, 1991) (Forster and Symons, *Cell* 48:211-220, 1987; Haseloff and Gerlach, *Nature* 328:596-600, 1988; Walbot and Bruening, *Nature* 334:196, 1988; Haseloff and Gerlach, *Nature* 334:585, 1988; Haseloff et al., U.S. Patent No. 5,254,678), hairpin ribozymes (Hampel et al., *Nucl. Acids Res.* 18:299-304, 1990, 5 and U.S. Patent No. 5,254,678), hepatitis delta virus ribozymes (Perrotta and Been, *Biochem.* 31:16, 1992), Group I intron ribozymes (Cech et al., U.S. Patent No. 4,987,071) and RNase P ribozymes (Takada et al., *Cell* 35:849, 1983); (see also, WO 95/29241, entitled "Ribozymes with Product Ejection by Strand Displacement"; and WO 95/31551, entitled "Novel Enzymatic RNA Molecules."

10 Cech et al. (U.S. Patent No. 4,987,071, issued January 22, 1991) disclosed the preparation and use of ribozymes which are based on the properties of the *Tetrahymena* ribosomal RNA self-splicing reaction. These ribozymes require an eight base pair target site and free guanosine (or guanosine derivatives). A temperature optimum of 50°C is reported for the endoribonuclease activity. The fragments that arise 15 from cleavage contain 5'-phosphate and 3'-hydroxyl groups and a free guanosine nucleotide added to the 5'-end of the cleaved RNA.

In contrast to the ribozymes of Cech et al., particularly preferred ribozymes of the present invention hybridize efficiently to target sequences at physiological temperatures, making them suitable for use *in vivo*, and not merely as 20 research tools (see column 15, lines 18 to 42, of Cech et al., U.S. Patent No. 4,987,071). Thus, particularly preferred ribozymes for use within the present invention include hairpin ribozymes (for example, as described by Hampel et al., European Patent Publication No. 0 360 257, published March 26, 1990) and hammerhead ribozymes. Briefly, the sequence requirement for the hairpin ribozyme is any RNA sequence 25 consisting of NNNBN*GUC(N)_x (Sequence ID Nos. 1-5) (where x is any number from 6 to 10, N*G is the cleavage site, B is any of G, C, or U, and N is any of G, U, C, or A). Representative examples of recognition or target sequences for hairpin ribozymes are set forth below in the Examples. Additionally, the backbone or common region of the hairpin ribozyme can be designed using the nucleotide sequence of the native hairpin 30 ribozyme (Hampel et al., *Nucl. Acids Res.* 18:299-304, 1990) or it can be modified to include a "tetraloop" structure that increases stability and catalytic activity (see Example 2 and Figure 3; see also Yu et al., *Virology* 206:381-386, 1995; Cheong et al., *Nature* 346:680-682, 1990; Anderson et al., *Nucl. Acids Res.* 22:1096-1100, 1994).

The sequence requirement at the cleavage site for the hammerhead 35 ribozyme is any RNA sequence consisting of NUX (where N is any of G, U, C, or A and X represents C, U, or A) can be targeted. Accordingly, the same target within the

hairpin leader sequence, GUC, is useful for the hammerhead ribozyme. Additional nucleotides of the hammerhead ribozyme or hairpin ribozyme are determined by the target flanking nucleotides and the hammerhead consensus sequence (*see* Ruffner et al., *Biochemistry* 29:10695-10702, 1990). This information, along with the sequences and
5 disclosure provided herein, enables the production of hairpin ribozymes of this invention. Appropriate base changes in the ribozyme are made to maintain the necessary base pairing with the target HCV sequences.

The ribozymes of this invention, as well as DNA encoding such ribozymes and other suitable nucleic acid molecules, described in more detail below,
10 can be chemically synthesized using methods well known in the art for the synthesis of nucleic acid molecules (*see e.g.*, Heidenreich et al., *J. FASEB* 70(1):90-6, 1993; Sproat, *Curr. Opin. Biotechnol.* 4(1):20-28, 1993). Alternatively, commercial suppliers such as Promega, Madison, Wis., USA, provide a series of protocols suitable for the production of nucleic acid molecules such as ribozymes.

15 Within one aspect of the present invention, ribozymes are prepared from a DNA molecule or other nucleic acid molecule (which, upon transcription, yields an RNA molecule) operably linked to an RNA polymerase promoter, *e.g.*, the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention are nucleic acid molecules, *e.g.*, DNA or cDNA, coding for the ribozymes of
20 this invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced *in vitro* upon incubation with the RNA polymerase and appropriate nucleotides. In a separate embodiment, the DNA may be inserted into an expression cassette, such as described in Cotten and Birnstiel, *EMBO J.* 8(12):3861-3866, 1989, and in Hempel et al., *Biochemistry*
25 28:4929-4933, 1989. A more detailed discussion of molecular biology methodology is disclosed in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989.

During synthesis, the ribozyme can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase
30 (Rossi et al., *Pharmac. Ther.* 50:245-254, 1991). Alternatively, the ribozyme can be modified to a phosphothio-analog for use in liposome delivery systems. This modification also renders the ribozyme resistant to endonuclease activity.

VECTORS

Use of ribozymes to treat HCV infection involves introduction of functional ribozyme to the infected cell of interest. This can be accomplished by either synthesizing functional ribozyme *in vitro* prior to delivery, or, by delivery of DNA
 5 capable of driving ribozyme synthesis *in vivo*.

More specifically, within other aspects of the invention the ribozyme gene may be constructed within a vector which is suitable for introduction to a host cell (e.g., prokaryotic or eukaryotic cells in culture or in the cells of an organism). Appropriate prokaryotic and eukaryotic cells can be transfected with an appropriate
 10 transfer vector containing the nucleic acid molecule encoding a ribozyme of this invention.

To produce the ribozymes with a vector *in vivo*, nucleotide sequences coding for ribozymes are preferably placed under the control of a eukaryotic promoter such as pol III (e.g., tRNA or VA-1 from adenovirus), CMV, SV40 late, or SV40 early
 15 promoters. Within certain embodiments, the promoter may be a tissue-specific promoter such as, for example, a liver-specific promoter such as the albumin promoter and the alphafetoprotein promoter (Feuerman et al., *Mol. Cell. Biol.* 9:4204-12, 1989; Camper and Tilghman, *Genes Develop.* 3:537-46, 1989); the alcohol dehydrogenase promoter (Felder, *Proc. Natl. Acad. Sci. USA* 86:5903-07, 1989); the Apolipoprotein B
 20 gene promoter (Das et al., *J. Biol. Chem.* 263:11452-8, 1988); the Coagulation protease factor VII gene promoter (Erdmann et al., *J. Biol. Chem.* 270:22988-96, 1995); the Fibrinogen gamma gene promoter (Zhang et al., *J. Biol. Chem.* 270:24287-91, 1995); the Glucokinase gene promoter (Williams et al., *Biochem. Biophys., Res. Comm.* 212:272-9, 1995); the Liver phosphofructokinase gene promoter (Levanon et al.,
 25 *Biochem. Mol. Biol. Int.* 35:729-36, 1995); the Phospho-Enol-Pyruvate Carboxy-Kinase ("PEPCK") promoter (Hatzogiou et al., *J. Biol. Chem.* 263: 17798-808, 1988; Benvenisty et al., *Proc. Natl. Acad. Sci. USA* 86:1118-22, 1989; Vaulont et al., *Mol. Cell. Biol.* 9:4409-15, 1989); or lymphoid-specific promoters. Ribozymes may thus be produced directly from the transfer vector *in vivo*.

30 A wide variety of vectors may be utilized within the context of the present invention, including for example, plasmids, viruses, retrotransposons and cosmids. Because HCV is an acute or chronic infection of the liver, vectors with hepatotropic properties are particularly preferred. Representative examples include adenoviral vectors (e.g., WO 94/26914, WO 93/9191; Yei et al., *Gene Therapy* 1:192-
 35 200, 1994; Kolls et al., *PNAS* 91(1):215-219, 1994; Kass-Eisler et al., *PNAS* 90(24):11498-502, 1993; Guzman et al., *Circulation* 88(6):2838-48, 1993; Guzman et

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al., *Cir. Res.* 73(6):1202-1207, 1993; Zabner et al., *Cell* 75(2):207-216, 1993; Li et al., *Hum Gene Ther.* 4(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci.* 5(10):1287-1291, 1993), adeno-associated type 1 ("AAV-1") or adeno-associated type 2 ("AAV-2") vectors (see WO 95/13365; Flotte et al., *PNAS* 90(22):10613-10617, 1993), hepatitis
5 delta vectors, live, attenuated delta viruses and herpes viral vectors (e.g., U.S. Patent No. 5,288,641), as well as vectors which are disclosed within U.S. Patent No. 5,166,320. Other representative vectors include retroviral vectors (e.g., EP 0 415 731; WO 90/07936; WO 91/02805; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218). For vectors without inherent
10 hepatotropism (e.g., AAV or retroviruses), cell tropism can be altered to specifically target these viruses to the liver. Methods of using such vectors in gene therapy are well known in the art, see, for example, Larrick, J.W. and Burck, K.L., *Gene Therapy: Application of Molecular Biology*, Elsevier Science Publishing Co., Inc., New York, New York, 1991 and Kreigler, M., *Gene Transfer and Expression: A Laboratory*
15 *Manual*, W.H. Freeman and Company, New York, 1990.

Further provided by this invention are vectors having more than one nucleic acid molecule encoding a ribozyme of this invention, each molecule under the control of a separate eukaryotic promoter (or, an Internal Ribosome Entry Site or "IRES"), or alternatively, under the control of single eukaryotic promoter.
20 Representative examples of other nucleic acid molecules which may be delivered by the vectors of the present invention include therapeutic molecules such as interferon (e.g., alpha, beta or gamma), as well as a wide variety of other cytokines or growth factors, and facilitators which assist or aid ribozymes in cleaving a target sequence by unwinding or otherwise limiting secondary folding which might otherwise inhibit the
25 ribozyme (see Example 4). These vectors provide the advantage of providing multi-functional therapy against HCV infection, preferably with the various therapies working together in synergy.

Host prokaryotic and eukaryotic cells stably harboring the vectors described above also are provided by this invention. Suitable host cells include
30 bacterial cells, rat cells, mouse cells, and human cells, for example, liver and blood cells.

DELIVERY

35 Within certain aspects of the invention, ribozyme molecules, or nucleic acid molecules which encode the ribozyme, may be introduced into a host cell utilizing

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a vehicle, or by various physical methods. Representative examples of such methods include transformation using calcium phosphate precipitation (Dubensky et al., *PNAS* 81:7529-7533, 1984), direct microinjection of such nucleic acid molecules into intact target cells (Acsadi et al., *Nature* 352:815-818, 1991), and electroporation whereby
5 cells suspended in a conducting solution are subjected to an intense electric field in order to transiently polarize the membrane, allowing entry of the nucleic acid molecules. Other procedures include the use of nucleic acid molecules linked to an inactive adenovirus (Cotton et al., *PNAS* 89:6094, 1990), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), microprojectile bombardment
10 (Williams et al., *PNAS* 88:2726-2730, 1991), polycation compounds such as polylysine, receptor specific ligands, liposomes entrapping the nucleic acid molecules, spheroplast fusion whereby *E. coli* containing the nucleic acid molecules are stripped of their outer cell walls and fused to animal cells using polyethylene glycol, viral transduction, (Cline et al., *Pharmac. Ther.* 29:69, 1985; and Friedmann et al., *Science* 244:1275, 1989), and
15 DNA ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989). In one embodiment, the ribozyme is introduced into the host cell using a liposome.

Within further embodiments of the invention, additional therapeutic molecules (e.g., interferon) or facilitators may be delivered utilizing the methods described herein. Such delivery may be either simultaneous to, or before or after, the
20 delivery of a ribozyme or vector expressing ribozymes.

PHARMACEUTICAL COMPOSITIONS

As noted above, pharmaceutical compositions also are provided by this
25 invention. These compositions contain any of the above described ribozymes, DNA molecules, vectors or host cells, along with a pharmaceutically or physiologically acceptable carrier, excipient, or diluent. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of
30 such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents.

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Pharmaceutical compositions of the present invention may also be prepared to contain, or express (*e.g.*, if a vector), one or more additional therapeutic molecules (*e.g.*, interferon) or facilitators.

• In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes, including for example intraarticularly, intradermally, intrahepatically, intramuscularly, intraperitoneally, intrathecally, intravenously (*e.g.*, into the portal vein), or subcutaneously. In addition, pharmaceutical compositions of the present invention may be placed within containers, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (*e.g.*, water, saline or PBS) which may be necessary to reconstitute the pharmaceutical composition

Pharmaceutical compositions are useful for both diagnostic or therapeutic purposes.

THERAPEUTIC METHODS

Methods of interfering with or preventing HCV viral replication, infectivity, or gene expression in a cell infected with HCV are also provided by this invention. Such methods require contacting the cell with an effective amount of ribozyme of this invention or, alternatively, by transducing the cell with an effective amount of vector having a nucleic acid molecule encoding the ribozyme. Effective amounts can be readily determined by those of skill in the art using well known methodology. When exogenously delivering the ribozyme, the RNA molecule can be embedded within a stable RNA molecule or in another form of protective environment, such as a liposome. Alternatively, the RNA can be embedded within RNase-resistant DNA counterparts. Cellular uptake of the exogenous ribozyme can be enhanced by attaching chemical groups to the DNA ends, such as cholesteryl moieties (Letsinger et al., *P.N.A.S., U.S.A.*, 1989).

In another aspect of the invention, the target cell is transduced under conditions favoring insertion of the vector into the target cell and stable expression of the nucleic acid encoding the HCV specific ribozyme. The target cell can include but is not limited to hepatocytes and lymphocytes. If the cell is transduced prior to HCV infection, infection of the target cell or its progeny can be prevented. Accordingly, this

aspect includes methods for interfering with or preventing HCV viral infection and/or replication in a cell by reacting the target RNA sequence with a ribozyme of this invention.

In one embodiment of this aspect of the invention, appropriate host cells
5 such as liver cells are removed from a subject, *e.g.*, a human patient, using methods well known in the art. The cells are then trypsinized and resuspended for *ex vivo* therapy. Within the cell or within the cells of an organism, a transfer vector as described above encoding one or more ribozymes is transfected into a cell or cells using methods described in Llewellyn et al., *J. Mol. Biol.* 195:115-123, 1987, and Hanahan, 166:557-
10 580, 1983. Inside the cell, the transfer vector replicates and the DNA coding for the ribozyme is transcribed by cellular polymerases to produce ribozymes which then inactivate HCV. Micromanipulation techniques such as microinjection also can be used to insert the vector into the cell so that the transfer vector or a part thereof is integrated into the genome of the cell. Transcription of the integrated material gives rise to
15 ribozymes which then inactivate HCV. The above methods are not intended to limit the invention, but merely to exemplify various means to effect the ribozyme therapy of this invention. Other methods are detailed in Anderson, *Science* 256:808-813, 1992.

For *ex vivo* therapy, the transduced cells can be reintroduced into the patient by hepatic artery injection under conditions such that the transduced cells will
20 integrate into the liver.

As used herein, the term "interfering with or preventing" HCV viral replication in a cell means to reduce HCV replication or production of HCV components necessary for progeny virus in a cell as compared to a cell not being transiently or stably transduced with the ribozyme or a vector encoding the ribozyme.
25 Simple and convenient assays to determine if HCV viral replication has been reduced include an ELISA assay for the presence, absence, or reduced presence of anti-HCV antibodies in the blood of the subject (Nasoff et al., *PNAS* 88:5462-5466, 1991), RT-PCR (Yu et al., in *Viral Hepatitis and Liver Disease* 574-477, Nishioka, Suzuki and Mishiro (Eds.); Springer-Verlag Tokyo, 1994) or liver function tests. Such methods
30 are well known to those of ordinary skill in the art. Alternatively, total RNA from transduced and infected "control" cells can be isolated and subjected to analysis by dot blot or northern blot and probed with HCV specific DNA to determine if HCV replication is reduced. Alternatively, reduction of HCV protein expression can also be used as an indicator of inhibition of HCV replication. A greater than fifty percent
35 reduction in HCV replication as compared to control cells typically quantitates a prevention of HCV replication.

DIAGNOSTIC METHODS

Detection and diagnosis of hepatitis C infection in humans has been somewhat problematic to date. The most commonly used methods involve detection of antibodies produced by the host against HCV structural proteins. Unfortunately, many patients remain seronegative for a variable length of time and frequently the eventual immune response can be difficult to detect. Furthermore, due to the considerable sequence variation in the HCV genome and mutations in the structural proteins, host-produced antibodies can vary significantly, requiring a large panel of antigens for accurate detection. For these reasons, a more reliable method for detection of infectious HCV would be useful, not only to test patient serum but also to screen the multitude of human-derived blood products purified for medical use.

HCV hairpin ribozymes can be applied to the detection and diagnosis of HCV infection. To accomplish this, a special reporter plasmid is generated which contains the HCV 5'-capsid sequence upstream of the *E. coli* lacZ gene (nucleotides 1302-4358, Genbank accession no. J01636). This plasmid is made via a two-step cloning process. First, HCV sequences containing the 5' UTR and capsid coding region are synthesized directly from RNA that was extracted from an HCV-positive patient serum sample. The purified viral RNA is then reverse transcribed and PCR amplified with the following primers: sense (starting at 5' end of 5' UTR) 5'-GCCAGCCCCC TGATGGGG - 3' (Sequence ID No. 6) and antisense (starting at 3' end of capsid coding region) 5'-CACCTGATAA GCGGAAGC - 3' (Sequence ID No. 7). The resulting blunt-end DNA is then ligated the unique *Sma* I site in pCMV β (Clontech, Palo Alto, CA). This first generation plasmid is designated pCMV-HCV- β . Second, to allow selection of this plasmid following transfection into mammalian cells, a neomycin resistance expression cassette, consisting of the SV40 early promoter driving the expression of the neomycin resistance gene, is constructed. This is accomplished by blunt-ligating a *Bam*HI fragment, containing the neomycin cassette obtained from pMAMneo-LUC (Clontech, Palo Alto, CA), into the unique *Sal*I site of pCMV-HCV- β . The resulting plasmid, pCMV-HCV- β -SV-neo expresses two independent RNAs. One containing the HCV target sites upstream of the lacZ coding sequence, and the other expressing neomycin resistance for positive selection.

To generate the reporter cell line, the human hepatocellular carcinoma cell line Huh7 (Yoo et al., *J. Virol.* 69:32-38, 1995), is co-transfected with pCMV-HCV- β -SV-neo and an HCV hairpin ribozyme expression plasmid, pLNT-Rz. G418-

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selected transfected Huh7 cells, containing both Rz and reporter plasmids, is then used for HCV infection diagnosis. Under normal conditions, expressed HCV Rz will cleave the HCV 5'UTR-capsid target located on the lacZ mRNA, resulting in the inhibition of β -galactosidase expression. When cells are challenged with a biological sample (e.g.,

5 patient serum samples or other blood products containing HCV, or tissue or cell samples taken from the liver), the presence of the HCV 5'UTR-capsid sequences coming from the replicating HCV will compete for the ribozyme, interfering with its ability to cleave the HCV-lacZ RNA. The result of this interference in Rz activity is an increased expression of β -galactosidase and these cells will stain blue by routine lacZ

10 staining. Thus, any patient serum (or other biological sample) which is positive for hepatitis C virus will cause these reporter cells to turn blue.

The following examples are offered by way of illustration, and not by way of limitation.

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EXAMPLESEXAMPLE 1

CRITERIA FOR HAIRPIN RIBOZYME SITE SELECTION

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Hairpin ribozymes suitable for use within the present invention preferably recognize the following sequence of RNA: NNNBNGUC(N)_x (Sequence ID Nos. 1-5) wherein the ribozyme is constructed so as to be complementary to the underlined sequences, and wherein B is C, G or U, and x is greater than or equal to 6
 10 nucleotides (*e.g.*, from 6 to 10 nucleotides). The sequence GUC must be conserved for all hairpin ribozymes described below. Other nucleotides ("N" as underlined above) preferably have a high degree of sequence conservation among different HCV strains in order to limit the need for multiple ribozymes against the same target site.

Briefly, sequence analysis of the complete genome of HCV-1 reveals
 15 112 suitable GUC hairpin ribozyme sites in the positive strand and 125 suitable sites in the negative strand. Due to the considerable sequence variability between different HCV genotypes, however, only 34 sites in the positive strand and 36 in the negative strand represent GUC's that are 95% conserved between all known strains. The nucleotides flanking the GUC (represented as "N" above) should also preferably contain
 20 a high degree of sequence conservation in order to limit the need to design multiple ribozymes against the same target site. To that end, 28 sites have been identified in the positive strand and 30 sites in the negative strand that have no more than one base mismatch in >85% of the known strains. These sites are provided below in Table I:

25

Table I

Hepatitis C Ribozymes Directed Against Both (+) and (-) Strand RNA

<u>RECOGNITION</u>		<u>LOCATION^b:</u>	<u>WITHIN:</u>	<u>SEQ. I.D.</u>
<u>RIBOZYME:</u>	<u>SEQUENCE^a:</u>			
HCV-1 (+) STRAND:				
CR1	ACCGG^GUCCUUUCUUG	190	5' UTR	8
CR2	UAGUG^GUCUGCGGAAC	146	5' UTR	9
CR3	CGGUG^GUCAGAU CGUU	424	Capsid	10
CR4	GAGCG^GUCGCAACCUC	506	Capsid	11

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CR4-7	GAGCG^GUCGCAACCU	506	Capsid	12
CR4-6	GAGCG^GUCGCAACC	506	Capsid	13
CR5	CUCCU^GUCACCCCGCG	635	Capsid	14
CR6	CUACU^GUCUUCACGCA	60	5' UTR	15
CR7	AAAGC^GUCUAGCCAUG	77	5' UTR	16
CR8	UGAGU^GUCGUGCAGCC	102	5' UTR	17
CR9	GUUGG^GUCGCGAAAGG	268	5' UTR	18
CR10	CGCUC^GUCGGCGCCCC	759	Capsid	19
CR11	AACUG^GUCGCCUACAA	1319	E1	20
CR12	AUAGG^GUCAGCGGUUG	2459	NS1	21
CR13	ACCUU^GUCACCACACU	2834	NS2	22
CR14	AUGCG^GUCCCCGGUCU	3958	NS3	23
CR15	CCCCG^GUCUUCACGGA	3965	NS3	24
CR16	GACGU^GUCCGUCAUAC	4610	NS3	25
CR17	CAUGU^GUCACCCAGAC	4712	NS3	26
CR18	AGACA^GUCGAUUUCAG	4724	NS3	27
CR19	GCGGU^GUCGCGCUCAC	4787	NS3	28
CR20	AGGUC^GUCACUAGCAC	5307	NS4	29
CR21	CUCCA^GUCCAAGCUCC	6329	NS4	30
CR22	GUUGA^GUCGUACUCCU	7491	NS5	31
CR23	UCUUG^GUCUACCGUGA	7556	NS5	32
CR24	ACAUG^GUCUAUGCCAC	7707	NS5	33
CR25	AAGGC^GUCCACAGUUA	7823	NS5	34
CR26	ACGUG^GUCUCCACCCU	8133	NS5	35
CR27	GGCCU^GUCGAGCUGCA	8506	NS5	36
CR28	CUACU^GUCCCAAGGGG	9134	NS5	37

HCV-1 (-) STRAND:

CNR1	GGAGU^GUCGCCCCCAA	23	"5'UTR"	38
CNR2	GGGGG^GUCCUGGAGGC	120	"5'UTR"	39
CNR3	CGGUC^GUCCUGGCAAU	183	"5'UTR"	40
CNR4	ACCCG^GUCGUCCAGGC	186	"5'UTR"	41
CNR5	UAGCA^GUCUCGCGGGG	247	"5'UTR"	42
CNR6	GCACG^GUCUACGAGAC	333	"5'UTR"	43
CNR7	CGGGG^GUCCGUGGGGC	674	"Capsid"	44
CNR8	ACGCC^GUCCUCCAGAA	821	"Capsid"	45

/8

CNR9	GAGCA [^] GUCAUUCGUGA	959	"E1"	46
CNR10	GCGGU [^] GUCCGCCCCC	3308	"NS2"	47
CNR11	GUCGA [^] GUCAGUUGAGU	4307	"NS3"	48
CNR12	CAGCC [^] GUCUCCGCUUG	4354	"NS3"	49
CNR13	AGAGC [^] GUCUGUUGCCA	4655	"NS3"	50
CNR14	UUGCA [^] GUCGAUCACCG	4700	"NS3"	51
CNR15	CCCGC [^] GUCAUAGCACU	4907	"NS3"	52
CNR16	GAGCA [^] GUCCUCAUUA	6242	"NS4"	53
CNR17	UACCC [^] GUCACGUAGUG	6649	"NS5"	54
CNR18	ACGUU [^] GUCGGUGGUCA	6665	"NS5"	55
CNR19	CAACC [^] GUCCUCUUUUU	7336	"NS5"	56
CNR20	UCAGG [^] GUCCCCCGGCU	7532	"NS5"	57
CNR21	GACCC [^] GUCGCUGAGAU	7547	"NS5"	58
CNR22	AGUCU [^] GUCAAAGGUGA	7763	"NS5"	59
CNR23	GGGGC [^] GUCAGCUUGCA	7873	"NS5"	60
CNR24	GUUGA [^] GUCAAAGCAGC	8273	"NS5"	61
CNR25	AAUUA [^] GUCAGGGGGCC	8398	"NS5"	62
CNR26	UAGUC [^] GUCAGCACGCC	8455	"NS5"	63
CNR27	GUGCA [^] GUCCUGGAGCU	8528	"NS5"	64
CNR28	ACCUA [^] GUCAUAGCCUC	8629	"NS5"	65
CNR29	AGUGU [^] GUCUAGGUCUC	8802	"NS5"	66
CNR30	GCGGG [^] GUCGGGCACGA	9303	"NS5"	67

^aRecognition sequence is written 5' to 3'. Cleavage occurs at ^.

^bLocation indicates nucleotide position of cleavage site (the "G" in the "GUC")

5 in either (+) strand, counting from 5' end, or (-) strand counting from 3' end.

Numbering is using HCV-1 strain.

EXAMPLE 2

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CONSTRUCTION OF HAIRPIN RIBOZYMES

Ribozyme genes (Rz) are designed by identifying highly sequence-conserved regions of the HCV genome that contain the obligatory GUC recognition signal. Rz's recognizing these sites are then designed on the basis of the nucleotide

sequence adjacent to the GUC recognition signal. More specifically, two single-stranded DNA oligonucleotides are chemically synthesized such that, when combined and converted into double-stranded DNA, they contain the entire hairpin ribozyme, including nucleotides complementary to the target site. In addition, restriction enzyme recognition sites may be placed on either end to facilitate subsequent cloning.

For example, in order to construct ribozyme CR4, which recognizes the target sequence: GAGCGGUCGCAACCUC (Sequence I.D. No. 11; Table I), in either the native or tetraloop hairpin ribozyme structure (Figure 3), the following oligonucleotides are prepared:

10

Sense oligo (Sequence I.D. No. 68):

*Bam*HI

5'-GCGGATCCGGAGGTTGCAGAAGCTCACCAGAGAAACACACG-3'

15 Universal antisense oligo (Sequence I.D. No. 69):

*Mlu*I

5'-GGGACGCGTACCAGGTAATATACCACAACGTGTGTTTCTCTGGT-3'

Universal tetraloop antisense oligo (Sequence I.D. No. 72):

20

*Mlu*I

5'-GGGACGCGTACCAGGTAATATACCACGGACCGAAGTCCGTGTGTTTCTCTGGT-3'

Restriction enzyme sites (*Bam*H I and *Mlu* I) are indicated in italics. Sequences complementary to the CR4 target site in HCV are underlined. The 16 bases of complementary sequence to allow annealing between the sense oligo and either the universal antisense or the universal tetraloop antisense oligonucleotide are in bold.

The appropriate oligonucleotides are annealed together and converted to double-stranded DNA using either Klenow DNA polymerase or Taq DNA polymerase. The resulting DNA is cleaved with restriction enzymes *Bam*HI and *Mlu*I, purified and cloned into vectors for *in vitro* transcription (pGEM7Z, Promega, Madison, WI; Figure 1) or for mammalian expression (pLNT; Figure 2).

Defective ribozymes (referred to as "disabled") for use as controls may be constructed as described above, with the exception that the sequence AAA, in loop 2, is changed to a UGC as shown in Figure 3.

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EXAMPLE 3

CONSTRUCTION OF HCV RIBOZYME MAMMALIAN EXPRESSION VECTORS

Plasmid pMJT (Yu et al., *Proc. Nat'l Acad. Sci. USA* 90:6340-6344, 1993; ATCC No. 75470), which contains the anti-U5 HIV ribozyme driven by the tRNA^{val} RNA pol III promoter, is digested with *Bam*HI and *Mlu*I, and the vector purified from the ribozyme fragment. The hepatitis C ribozyme genes, as described above, are excised from the pGem7Z vector (Figure 1) with *Bam*HI and *Mlu*I, purified, and ligated into the empty pMJT vector. The resulting vector is designated pLNT-Rz (see Figure 2), and contains the Moloney LTR driving the neomycin resistance gene and the tRNA^{val} RNA pol III promoter driving expression of the ribozyme.

EXAMPLE 4

IN VITRO CLEAVAGE ASSAYS

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Ribozyme genes are cloned into *in vitro* transcription vectors (pGEM-7Z, Promega, Madison, Wis.) and transcribed *in vitro* by T7 RNA polymerase. Following transcription, reactions are treated with DNase and the ribozymes are purified by denaturing polyacrylamide gel electrophoresis.

20

The HCV substrates are as follows: short 5'UTR substrate HCV-1 nucleotides 1-185, short capsid substrate HCV-1 nucleotides 331-698 and long substrate HCV-1 nucleotides 1-698. Substrates are transcribed *in vitro* in the presence [α -³²P]UTP and purified by denaturing polyacrylamide gel electrophoresis. The *in vitro* cleavage reactions are carried out by incubating 40 nM ribozyme with 200 nM substrate at 37°C for 0 to 60 minutes in 12 mM MgCl₂/2 Mm spermidine/40 mM Tris-HCl, pH 7.5. Reactions are terminated by the addition of loading buffer (7 M urea/bromophenol blue/xylene cyanol). Products of the cleavage reactions are resolved by electrophoresis on 15% acrylamide/7 M urea gels and analyzed by autoradiography.

25

The data in Figure 4 indicates that the CR2 Rz cleaves the short 5' UTR 185-nucleotide target RNA in a time dependent manner. Interestingly, the same Rz could not cleave a larger HCV RNA substrate that contained the entire 5' UTR (long HCV-1 substrate), presumably due to a highly folded secondary structure that is not present in the short substrate. In fact, the larger HCV substrate RNA could be cleaved by the Rz *in vitro*, only if the RNA substrate is heat denatured before the cleavage reaction (data not shown).

35

Occasionally, secondary structure in the target RNA is extensive enough to inhibit the binding and cleavage activities of hairpin ribozymes. One example is the 5'UTR of HCV, where RNA folding inhibits the activity of CR2. Thus, "facilitator" RNA molecules can be designed to enhance the activity of ribozymes targeted to highly folded structures. Briefly, facilitator molecules are RNA's that are engineered to be complimentary to regions of the target RNA flanking the ribozyme target site. When bound to the target RNA, these molecules help to relax the secondary structure, thus enhancing the binding, and therefore the activity, of the ribozyme.

Three facilitators are designed to disrupt the folded structure in the 5'UTR and were tested for their ability to enhance the activity of CR2. In particular, the following facilitators may be utilized: HCV269, HCV269B and HCV236 and are complimentary to bases 245-268, 242-268 and 217-235 of the 5' UTR of HCV-1 respectively. Facilitator RNAs are synthesized *in vitro*, added at a concentration of 40 nM to *in vitro* cleavage reactions containing CR2 and the long HCV-1 substrate, and cleavage reactions are monitored over 3 hours (Figure 5). In the absence of any facilitator, less than 1% of the long HCV-1 substrate was cleaved over a period of 3 hours (Figure 5, filled squares), consistent with previous results. Addition of either facilitator molecule, HCV269 or HCV269B, enhances the substrate cleavage 3- to 3.5-fold over the control (Figure 5, filled triangles and filled diamonds). The stimulation seen with either single facilitator is similar to that seen for the reaction without facilitator, when ribozyme and substrate are heated to 90°C for 1 minute prior to incubation at 37°C (Figure 5, filled circle). When the facilitators HCV269B and HCV236 are added in combination, the reaction was stimulated 9-fold over the control reaction (Figure 5, open squares), indicating an additive to synergistic effect. These results indicate that ribozymes inhibited by secondary structure in the substrate (such as CR2) can become active against full length substrates when used with appropriately designed facilitator molecules.

Three additional Rz directed against the HCV 5' UTR (CR6, CR7, and CR8) are also tested on the 185-nucleotide short 5' UTR substrate. As indicated by the data in Figure 6, these Rz could only cleave the short 5' UTR substrate when it is heat denatured, while cleavage of the short 5'UTR substrate by the CR2 Rz, as before, is independent of heat denaturation. Consistent with the role of secondary structure in the availability of RNA for Rz cleavage, the target sequences for CR6, CR7, and CR8 are in a location that allows extensive secondary structure, even in the short 5' UTR substrate.

A potential Rz cleavage site in a highly conserved region of the HCV Capsid protein, not containing as much secondary structure as the 5' UTR, is next

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tested. Like the previously tested CR2 Rz, the Capsid-targeted CR4-8 Rz cleaves a short HCV capsid substrate RNA *in vitro*, without heat denaturation (Figure 7). Since the catalytic efficiency of Rz may be limited by the dissociation constant, and thus the size of the target sequence recognized, ribozymes are also generated and tested that

5 cleave the same site as CR4-8, but recognize a target sequence that lacks one nucleotide (CR4-7) or two nucleotides (CR4-6) compared with the CR4-8 Rz. This is accomplished by changing the length of Helix 1 (Figure 3). The data in Figure 7 indicate that CR4-7 and CR4-6 are not more active than CR4-8, in fact cleavage by CR4-8 may be more efficient.

10 As indicated by the data in Figure 8, CR-4 is capable of cleaving the long HCV substrate RNA, containing the extensive secondary structure, without heat denaturation. The autoradiogram in Figure 8 is overexposed to reveal the low molecular weight cleavage product, but lighter exposures reveal the expected higher molecular weight fragment (additional exposure not shown). As with the short

15 substrate, cleavage by CR4-8 is identical with, or slightly more effective than, that of either CR4-7 or CR4-6.

EXAMPLE 5

20 CONSTRUCTION OF VECTORS, AND TESTING OF GENE EXPRESSION IN TISSUE CULTURE

As described in more detail below, reporter systems are developed to evaluate the effect of Rz expression on the expression of genes containing HCV Rz target sequences.

25

A. Construction of Vectors

Construction of several expression vectors is described herein (Figure 9). The HCV reporter plasmid pPur-HCV (Figure 9B) is constructed as follows: HCV sequences containing the 5'UTR and capsid coding region are synthesized directly from

30 RNA that is extracted from an HCV-positive patient serum sample. The purified viral RNA is then reverse transcribed and PCR amplified with the following primers: sense (starting at 5' end of 5' UTR) 5'-GCCAGCCCCC TGATGGGG - 3' (Sequence ID No. 6) and antisense (starting at 3' end of capsid coding region) 5'-CACCTGATAA GCGGAAGC - 3' (Sequence ID No. 7). The resulting blunt-end DNA is then ligated

35 into plasmid pPur (Clontech, Palo Alto, CA; Figure 9A) that has been digested with *Xba*I and blunt-ended with Klenow DNA polymerase. The HCV reporter retroviral

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vector pLNL-Pur-HCV (Figure 9D) is constructed by purifying the 2065 bp PvuII/XbaI fragment from pPur-HCV, which contains the SV40 early promoter, the puromycin resistance coding region and the HCV 5'UTR and capsid sequences. The fragment is blunt-ended with Klenow and cloned into plasmid pLNL6 (Bender et al., *J. Virol.* 61:1639-1646, 1987; Figure 9C) that has been digested with *Hind*III and blunt-ended with Klenow. Both resulting HCV reporter plasmids will then produce an RNA transcript, via SV40 early promoter, that contains the HCV 5' UTR and capsid sequences on the same RNA transcript as the coding region for puromycin resistance. Each HCV ribozyme is expressed on a separate retroviral vector (pLNT-Rz) via the tRNA^{val} pol III promoter. Active HCV ribozymes will cleave the Pur-HCV RNA, resulting in a cell sensitive to puromycin.

B. Co-transfection and Assay of Ribozymes

The ability of Rz to inactivate RNA containing HCV sequences is determined by co-transfection of a Rz-expressing plasmid (pLNT-Rz) with plasmids expressing an mRNA that contains HCV target sequences and codes for puromycin resistance (pPur-HCV).

Briefly, HeLa or HT1080 cells are co-transfected with pPur-HCV and various pLNT-Rz constructs using standard calcium phosphate methods. DNA molar ratios for the HCV:Rz co-transfections is 1:10, using empty pLNT vector to maintain total DNA at 20 µg. Ribozymes tested included two anti-HCV ribozymes: CR2 (against the 5' UTR) and CR4 (against capsid), and one disabled anti-HBV ribozyme, dBR1, included as a negative control. Cells are selected with 1 µg/ml puromycin starting 24 hours post transfection, and continued for up to two weeks. Puromycin-resistant colonies are visualized by crystal violet staining and counted. Ribozyme expression within the transfected cells is verified by RNase protection. Using radiolabeled antisense CR4 RNA as the RNase protection probe, the expected protected fragments are 64nt for CR4, 42nt for CR2 and 32nt for dCR4 (Figure 10; RNase Protection Assay Kit is available from Promega, Madison, WI).

Expression of either Rz CR2 or Rz CR4 results in a 70-90% reduction in puromycin-resistant colonies as compared to the dBR1 negative control (Figure 11). Results are similar in two human cell lines: HeLa (Figure 11A) and HT1080 (Figure 11B). To verify that the ribozymes are exerting their effect via the HCV sequences, similar co-transfections are performed with a pPur plasmid lacking the HCV target sequence. Under these conditions, co-expression of CR2 or CR4 has no effect on the

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number of puromycin-resistant colonies (data not shown), indicating that the ribozymes are targeting the HCV sequence.

To determine if the activity of CR4 is dose-dependent, co-transfections are performed with varying amounts of pLNT-CR4. DNA molar ratios for the HCV:Rz co-transfections ranged from 1:1 to 1:10, using empty pLNT vector to maintain total DNA at 20 µg. As a negative control, disabled CR4 (dCR4) is also co-transfected at each molar ratio. Again, cells are selected with 1 µg/ml puromycin, starting 24 hours post transfection, and continued for up to two weeks. Puromycin-resistant colonies are visualized by crystal violet staining and counted.

Expression of increasing amounts of CR4, but not dCR4, resulted in a dose-dependent reduction in the number of puromycin-resistant colonies in HT1080 cells (Figure 11C). Together, the results presented in Figure 11 indicate that co-expression of the HCV Rz interferes with the expression of the puromycin resistance marker, and appears to exert its effect through cleavage of the HCV target site.

C. Protection Assays

Stable expression of HCV Rz's is also tested to determine if it could protect a cell from infection with a related virus that shares some of the properties of HCV. Like HCV, retroviruses also have a positive, single-stranded, RNA genome. Thus, retroviral vector, pLNL-Pur-HCV (Figure 9D) is constructed to contain an HCV Rz target sequence in tandem with the Puromycin resistance gene. Polyclonal cell lines that stably express each of the ribozymes via the tRNA^{val} promoter are established in the human hepatocellular carcinoma cell line, HepG2, by transduction with retroviral vector, pLNT-Rz (Figure 2), followed by G418 selection. Helper-free amphotropic retroviral vector is then produced in PA317 cells, using pLNL-Pur-HCV (Figure 9D), which contains within its (+) strand RNA genome, the HCV 5' UTR and capsid sequences. The HepG2-Rz cell lines are then "challenged" by transduction with the HCV sequence-containing retroviral vector. To determine the level of transduction, cells are selected with 1 µg/ml puromycin starting 24 hours post transduction, and continued for up to two weeks. Puromycin-resistant colonies are stained and counted (see Figure 12 for a schematic representation of the experiment).

Stable expression of both the CR2 Rz or the CR4 Rz "protected" the cell from incoming retroviral vector containing HCV Rz target sequences, compared with the disabled Rz control dCR4 (Figure 13). Similar results are also shown in HeLa cells (data not shown). Together, these results confirm the previous result that the HCV ribozymes could function within human liver cells. Furthermore, these results indicate

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that stable expression of HCV Rz's can inhibit "infection" with viruses that contain positive-stranded RNA genomes.

5

EXAMPLE 6

TESTING ADDITIONAL HCV RIBOZYME TARGET SITES IN TISSUE CULTURE

The reporter systems, as described thus far, are suitable only for the analysis of ribozymes targeted to the 5'UTR and capsid regions of HCV. In order to test the additional target sites listed in Table I, *in vivo* assay systems can be designed which are capable of testing the cleavage by ribozymes of any site within the either the (+) or (-) strand RNA.

15

A. Analyzing Ribozyme Cleavage in the (+) Strand

The (+) RNA strand of HCV contains the open reading frame responsible for the translation of the 3011 amino acid polyprotein. A western blot assay can be developed, using human antiserum from HCV-infected patients, to detect translation of the nucleocapsid (or capsid) protein in transfected cells (Figure 14). Briefly, transfection of HT1080 cells with pPur-HCV (see Figure 9B) results in the transcription of an mRNA containing the HCV 5'UTR and capsid sequences. It has previously been demonstrated the 5'UTR of HCV can act as an Internal Ribosome Entry Site (IRES), which allows protein translation to initiate from within an RNA (Wang et al., *J. Virology* 67:3338-3344, 1993). Thus, the transfected cells translate the capsid sequences and the 21 kD capsid protein is visualized by western blotting (Figure 14). In this experiment, only the first 1000 nucleotides of HCV are present in the mRNA, resulting in the translation of just the capsid protein. However, when more of the HCV sequence is included (anywhere up to the total 9500 basepairs), a larger polyprotein is translated and the resulting protein detected by our western blot assay is correspondingly larger (not shown). Since the capsid coding region is the first region of the HCV polyprotein to be translated, its detection can be used to visualize translation of any length polyprotein, up to the full size HCV polyprotein of nearly 350 kD. Ribozymes targeted anywhere on the (+) strand RNA would cleave the HCV mRNA, resulting in the translation of a polyprotein smaller than that translated in the absence of ribozyme. This assay can not only detect ribozyme activity, it can also be used to

26

quantitatively compare the activity of various ribozymes targeted to different sites anywhere on the (+) strand.

B. Analyzing Ribozyme Cleavage in the (-) Strand

5 Since there is currently no cell culture system that supports HCV replication, and therefore the production of HCV (-) strand RNA, a luciferase reporter system can be developed to analyze the effect of ribozymes targeted against the (-) strand. Briefly, fragments of the (-) strand are cloned into the unique *Hind* III site upstream of the luciferase coding region in the pGL3 vector (Figure 15; Promega,
10 Madison, WI). These vectors, when transfected into mammalian cells, transcribe mRNA, via the SV40 early promoter, that contains the fragment of the (-) strand RNA upstream of the luciferase coding region. Ribozymes directed against the (-) strand RNA are first cloned into pLNT-Rz (see Figure 2) and then co-transfected with the HCV-luciferase reporter plasmid. Ribozymes capable of cleaving the (-) strand *in vivo*
15 will thus result in a decrease in luciferase activity after transfection. Luciferase activity is measured by the Luciferase Assay System (Promega, Madison, WI). Similar to the assay system for testing ribozymes on the (+) strand described above, this luciferase-based assay not only can detect ribozyme activity, but can also be used to quantitatively compare the activity of various ribozymes targeted to different sites anywhere on the (-)
20 strand.

EXAMPLE 7

CONSTRUCTION OF ADENOVIRUS AND AAV
RIBOZYME DELIVERY VECTORS

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A. Construction and Purification of Adenoviral Delivery Vectors

Recombinant replication-deficient (E1 deletion) adenoviral vectors (Av) containing HCV specific ribozyme genes are constructed by homologous recombination of shuttle plasmid (pAvCRz.SY; Figure 16) with Ad dl-327 (Jones and Shenk, *Cell*
30 13:181-188, 1978). Briefly, the shuttle plasmid is a pBR322-based plasmid consisting of the following elements: (1) Ad5 sequence 1-452 (Genebank accession no. M73260; containing the left inverted terminal repeat, encapsidation signals and the Ela enhancer), artificial *Xba*I, *Bam*HI and *Xho*I sites, (2) CMV immediate/early gene promoter and enhancer (from pCMV β expression vector, Clontech, Palo Alto, CA;
35 Boshart et al., *Cell* 41:521-530, 1985), artificial *Bam*HI and *Xho*I sites, SV40 splice donor/splice acceptor sequence (from pCMV β expression vector, Clontech, Palo Alto,

CA), artificial multiple cloning sites include *Bam*HI, *Not*I, *Bgl*II, *Eco*RI, *Asc*I, *Not*I, *Bam*HI sites in a contiguous arrangement, SV40 polyadenylation (from pCMV β expression vector, Clontech, Palo Alto, CA), artificial *Bam*HI, *Sal*I and *Clal* sites; and (3) an Ad5 sequence used for homologous recombination (Ad5 sequences 3328-5788).

5 HCV-specific ribozyme genes (such as CR2 and CR4 and others) or reporter genes (such as *E. coli* β -galactosidase gene from pCMV β expression vector, Clontech, Palo Alto, CA) are cloned into the shuttle plasmid via the multiple cloning site (Figure 16). Recombinant adenoviral vectors are plaqued and purified from 293 cells co-transfected with both the shuttle plasmid and large *Clal*-fragment of dl327. The resulting Av are

10 plaque-purified for at least 2 more times as performed routinely (Yei et al, 1994, *Human Gene Therapy* 5:731-744). Av can be propagated in 293 cells and purified to high titer preparation before use in evaluating the function of anti-HCV Rz.

Since intravenous use in animals requires the preparation of large amounts of highly purified vector (e.g., Adenoviral vector preparations), within certain

15 embodiments of the invention FPLC may be utilized to purify the relevant viral vector. For example, as shown in Figure 17, wild-type Adenovirus, used here as a model for Adenoviral vector, can be purified from clarified cell lysates by salt gradient elution of ion-exchange column chromatography. The virus, as measured by an infectivity assay, eluted as a sharp peak at approximately 14-15 ml. It is estimated that the virus is at

20 least 90% pure and results in yields that are comparable to other, less scalable, purification techniques, such as CsCl gradient centrifugation.

B. Construction of AAV Delivery Vectors

Recombinant AAV (rAAV) vectors containing HCV specific ribozymes

25 can also be constructed. Potential ribozymes include, but are not limited to CR2 and CR4. Briefly, within preferred embodiments the ribozyme(s) are placed under the control of an CMV promoter, and a selectable marker, such as neomycin resistance, is placed under the control of an SV40 early promoter (Figure 18). The ribozyme can contain the bovine growth hormone (BGH) polyadenylation signal and can also contain

30 a cleavage cassette which allows for 50% of the ribozyme molecules to be non-polyadenylated. The neo mRNA can be polyadenylated by the SV40 polyadenylation signal. The entire cassette can be flanked by AAV ITRs to allow for packaging into an AAV particle (Figure 18). To package the AAV vector, poly-l-lysine is cross-linked to the surface of adenovirus type 5 and the positively charged virus is bound to DNAs for

35 rAAV and the AAV helper plasmid pAAV/Ad (pAAV/Ad contains all the necessary AAV complementation functions for packaging of AAV genomes; Samulski et al., *J.*

28

Virology 63:3822-3828, 1989). Subsequently, the adenovirus poly-lysine complex is used to infect HeLa cells and approximately 48 hours post-infection the cells are harvested and lysed by freeze-thaw to release packaged rAAV. The cell lysate is then heated to 56°C to inactivate replication competent adenovirus and the rAAV is then
5 purified or used crude to titer and transduce liver cells.

EXAMPLE 8

IN VITRO CLEAVAGE OF HCV NEGATIVE 10 GENOMIC STRAND RNA BY RIBOZYMES

Ribozyme genes which encode target HCV (-) strand RNA were synthesized and cloned into *in vitro* transcription vectors (pGEM-7Z, Promega, Madison, Wis.) as described in Example 2. The resultant plasmids, designated as
15 pGem7Z-(-) ribozyme, were linearized with *Nsi*I (immediately downstream of the cloned gene) and transcribed *in vitro* by T7 (Welch et al., *Gene Therapy* 3:994-1001, 1996). The reactions were treated with RQ1 DNase, according to information provided by the manufacturer (Promega) and the transcripts were gel purified.

HCV negative strand RNA substrates were also obtained by *in vitro*
20 transcription, using the plasmid pGEM7-HCV5' for long substrate synthesis (471 bp in length). The short substrate was made by annealing 2 oligos: one with cleavage site and one with T7 promoter, and filled in with T4 DNA polymerase as described previously (Welch et al., 1996, *supra*)

In vitro cleavage assays were performed essentially as described above.
25 Results are shown in Figures 19 and 20. Briefly, the blots in Figures 19 and 20 clearly indicate that the CNR3 Rz cleaves the short (19) and long (20) negative strand HCV RNA substrate.

EXAMPLE 9

CONSTRUCTION OF ADENOVIRUS AND AAV RIBOZYME DELIVERY VECTORS 30 WITH VARIOUS PROMOTERS

A. Construction of Adenoviral Vectors

35 Replication-deficient adenoviral vectors expressing the catalytically active Rz (CR4) identified in Example 5 driven by various promoter were constructed

essentially as described in Example 7, in order to contain a tRNA^{val} promoter (Yu et al., *PNAS* 84:1005, 1993) or CMV promoter or both. The resulting plasmids are illustrated in Figure 21: pAvC-Rz express Rz under CMV promoter, pAvM-Rz express Rz under control of tRNA^{val} promoter and pAvCM-Rz express Rz from both CMV and tRNA^{val} promoters.

B. Construction of AAV Vectors

The CR4 Rz gene was also cloned into pAMFT.dBam plasmid to generate rAVV vectors expressing CR4 from tRNA^{val} promoter. Briefly, pAMFT.dBam (Figure 22) is a recombinant plasmid carrying 1) 5' and 3' inverted terminal repeats (ITR) of adeno-associated viral genome; 2) cassette for transcription of Rz gene via tRNA^{val} promoter; 3) neomycin resistance marker driven by MMLV LTR. The resultant plasmid for generating AAV vectors that express CR4 ribozyme under control of tRNA^{val} promoter is designated as pAAVM-CR4 (Figure 21).

EXAMPLE 10

EFFECT OF CR4 RZ IN REDUCING HCV GENE EXPRESSION

CR4 Rz activity in reducing HCV gene expression was tested in tissue culture. Briefly, CR4 Ribozyme was expressed from pAvC-CR4 described in Example 9. Western blot to detect HCV core antigen was used to evaluate the effectiveness of ribozyme in inhibiting the intracellular synthesis of HCV capsid in tissue culture. In particular, HT1080 cells were co-transfected by pPur-HCV (see Figure 23) and pAvC-CR4 at a molar ratio of 1:10 or 1:20 using method described in Example 5. Ribozymes tested included CR4 and disabled CR4 (dCR4). Cells were harvested at 24 hours post transfection and processed for HCV core Western blot.

As shown in Figure 24, co-transfection of pAvC-CR4 at the molar ratios tested (1:10 and 1:20) results in nearly complete shut off of HCV core expression while disabled form of CR4 (dCR4) had no effect (Figure 24).

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EXAMPLE 11EFFECT OF DIFFERENT PROMOTER/VECTOR COMBINATIONS
IN REDUCING HCV GENE EXPRESSION

5 Effect of different promoter/vector combination on CR4 Rz activity in
reducing HCV gene expression was further tested in tissue culture. Briefly, the studies
were done in a way similar to that described in Example 10, except that CR4 Rz was
expressed from different promoter/vector constructs. Expression of CR4 from different
constructs all resulted in reduced HCV core expression (Figure 25, panel A), as seen in
10 Example 10. However, pAAVM-CR4 (adeno-associated virus DNA backbone with
tRNA^{val} pol III promoter expressing CR4 Rz) shows the greatest efficacy (Figure 25-A).

 The activity of pAAVM-CR4 was also titrated for reducing viral core
expression by co-transfected the cells with HCV:Rz ratio at 1:0, 1:0.5, 1:1, 1:5, 1:10
and 1:20. Complete inhibition of core protein expression even at 1:1 ratio. Significant
15 reduction seen at 1:0.5 ratio (Figure 25, panel B).

EXAMPLE 12*IN VIVO* EXPRESSION OF Rz CR4 IN LIVER CELLS

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 The ability of recombinant adenovirus to deliver and express a ribozyme
gene in human liver cells was evaluated essentially as follows. Briefly, adenovirus was
generated that carries the CR4 gene driven by the CMV promoter (AvC-CR4). Human
hepatoma cells (Huh7) were transduced with vector at m.o.i. of 0, 1, 5, 10 or 50 and
25 cellular RNA was harvested at 1 or 3 days post transduction.

 RNase protection assay (Promega, Madison, WI) was then performed in
order to measure the expression of ribozyme. Briefly, as shown in Figure 26, CR4
ribozyme was expressed equally at day 1 and day 3 post transduction and expression
levels indicated a dose-response dependent on the amount of virus transduced. These
30 results indicate that adenovirus can be used to deliver and direct expression of a
ribozyme gene in human liver cells.

EXAMPLE 13**INFECTIVITY OF PRIMARY NORMAL HUMAN HEPATOCYTES WITH EITHER
ADENOVIRAL OR ADENO-ASSOCIATED VIRAL VECTOR**

5 The infectivity of primary normal human hepatocytes to the AV and
AAV vector constructs were tested essentially as described below. Briefly, primary
normal human hepatocytes were infected with either AV or AAV vector that carrying
beta-galactosidase gene at various MOI. At 24 hours post infection, cells were
processed for lacZ staining. Number of cells stained blue due to transduction of the
10 vectors were counted.

Results are shown in Figure 27. Briefly, both AV and AAV can
efficiently infect and transduce the primary hepatocytes in culture with 100%
infectivity.

15 From the foregoing, it will be appreciated that, although specific
embodiments of the invention have been described herein for purposes of illustration,
various modifications may be made without deviating from the spirit and scope of the
invention. Accordingly, the invention is not limited except as by the appended claims.

32

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Barber, Jack R.
Welch, Peter J.
Tritz, Richard
Yei, SoonPin
Yu, Mang

(ii) TITLE OF INVENTION: HEPATITIS C VIRUS RIBOZYMES

(iii) NUMBER OF SEQUENCES: 73

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: SEED and BERRY LLP
(B) STREET: 6300 Columbia Center, 701 Fifth Avenue
(C) CITY: Seattle
(D) STATE: Washington
(E) COUNTRY: USA
(F) ZIP: 98104-7092

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: McMasters, David D.

33

(B) REGISTRATION NUMBER: 33.963

(C) REFERENCE/DOCKET NUMBER: 480124.403PC

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (206) 622-4900

(B) TELEFAX: (206) 682-6031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

NNNBNGUCNN NNNN

14

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

34

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

NNNBNGUCNN NNNNN

15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NNNBNGUCNN NNNNN

16

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

NNNBNGUCNN NNNNNNN

17

35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

NNNBNGUCNN NNNNNNNN

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCAGCCCCC TGATGGGG

18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs

36

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACCTGATAA GCGGAAGC

18

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCGGGUCCU UUCUUG

16

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

UAGUGGUCUG CGGAAC

16

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGGUGGUCAG AUCGUU

16

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

38

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAGCGGUCGC AACCUC

16

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGCGGUCGC AACCU

15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGCGGUCGC AACC

14

39

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CUCCUGUCAC CCCGCG

16

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CUACUGUCUU CACGCA

16

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs

40

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AAAGCGUCUA GCCAUG

16

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

UGAGUGUCGU GCAGCC

16

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

41

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GUUGGGUCGC GAAAGG

16

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGCUCGUCGG CGCCCC

16

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:⁴²

AACUGGUCGC CUACAA

16

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AUAGGGUCAG CGGUUG

16

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ACCUUGUCAC CACACU

16

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(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AUGCGGUCCC CGGUCU

16

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCCCGGUCUU CACGGA

16

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs

- 44
- (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GACGUGUCCG UCAUAC

16

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CAUGUGUCAC CCAGAC

16

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

43

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AGACAGUCGA UUUCAG

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(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCGGUGUCGC GCUCAC

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(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

46

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AGGUCGUCAC UAGCAC

16

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CUCCAGUCCA AGCUCC

16

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GUUGAGUCGU ACUCCU

16

47

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

UCUUGGUCUA CCGUGA

16

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ACAUGGUCUA UGCCAC

16

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs

48

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AAGGCGUCCA CAGUUA

16

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ACGUGGUCUC CACCCU

16

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

44

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GGCCUGUCGA GCUGCA

16

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CUACUGUCCC AAGGGG

16

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GGAGUGUCGC CCCCAA

16

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGGGGGUCCU GGAGGC

16

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CGGUCGUCCU GGCAAU

16

51

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ACCCGGUCGU CCAGGC

16

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

UAGCAGUCUC GCGGGG

16

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs

52

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCACGGUCUA CGAGAC

16

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CGGGGGUCCG UGGGGC

16

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

53

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

ACGCCGUCCU CCAGAA

16

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GAGCAGUCAU UCGUGA

16

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

54

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCGGUGUCCG CCCCCC

16

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GUCCAGUCAG UUGAGU

16

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CAGCCGUCUC CGCUUG

16

55

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

AGAGCGUCUG UUGCCA

16

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

UUGCAGUCGA UCACCG

16

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs

56

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

CCCGCGUCAU AGCACU

16

(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

GAGCAGUCCU CAUUA

16

(2) INFORMATION FOR SEQ ID NO:54:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

57

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

UACCCGUCAC GUAGUG

16

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

ACGUUGUCGG UGGUCA

16

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

58

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CAACCGUCCU CUUUUU

16

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

UCAGGGUCCC CCGGCU

16

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GACCCGUCGC UGAGAU

16

59

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

AGUCUGUCAA AGGUGA

16

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GGGGCGUCAG CUUGCA

16

(2) INFORMATION FOR SEQ ID NO:61:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs

- (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

GUUGAGUCAA AGCAGC

16

(2) INFORMATION FOR SEQ ID NO:62:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

AAUUAGUCAG GGGGCC

16

(2) INFORMATION FOR SEQ ID NO:63:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

61

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

UAGUCGUCAG CACGCC

16

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GUGCAGUCCU GGAGCU

16

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:⁶²

ACCUAGUCAU AGCCUC

16

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

AGUGUGUCUA GGUCUC

16

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

GCGGGGUCGG GCACGA

16

(2) INFORMATION FOR SEQ ID NO:68:

63

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

GCGGATCCGG AGGTTGCAGA AGCTACCAG AGAAACACAC G

41

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

GGGACGCGTA CCAGGTAATA TACCACAACG TGTGGTTTCT CTGGT

45

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs

64

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

GGAGGUUGCA GAAGCUCACC AGAGAAACAC ACGUUGUGGU AUAUUACCUG GUA 53

(2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

GAGCGGUCGC AACCUC

17

(2) INFORMATION FOR SEQ ID NO:72:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 53 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

65

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GGGACGCGTA CCAGGTAATA TACCACGGAC CGAAGTCCGT GTGTTTCTCT GGT 53

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 62 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GGAGGUUGCA GAAGCUCACC AGAGAAACAC ACGGACUUCG GUCCGUGGUA UAUUACCUGG 60

UA 62

66

Claims

We claim:

1. A ribozyme having the ability to inhibit replication, infectivity, or gene expression of a hepatitis C virus.
2. The ribozyme according to claim 1 wherein said ribozyme cleaves HCV negative strand genomic RNA.
3. The ribozyme according to claim 1 or 2 wherein said ribozyme is a hairpin ribozyme.
4. A nucleic acid molecule encoding a ribozyme according to any one of claims 1 to 3.
5. The nucleic acid molecule of claim 4 wherein the nucleic acid molecule is DNA or cDNA.
6. A host cell containing the ribozyme or nucleic acid molecule according to any one of claims 1 to 5.
7. A vector, comprising a promoter operably linked to the nucleic acid molecule according to claim 4 or 5.
8. The vector according to claim 7 wherein said promoter is a polIII or CMV promoter.
9. The vector according to claim 7 wherein said vector is a plasmid, a viral vector, retrotransposon or a cosmid.
10. The vector according to claim 7 wherein said viral vector is a recombinant adenoviral or retroviral vector.
11. A host cell containing the vector according to any one of claims 7 to 10.

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12. A method for producing a ribozyme, the ribozyme being able to inhibit hepatitis C viral infection, replication, or gene expression in a cell, comprising providing DNA encoding a ribozyme according to any one of claims 1 to 3 under the transcriptional control of a promoter, and transcribing the DNA to produce said ribozyme.

13. The method according to claim 12, further comprising the step of purifying the ribozyme.

14. The method according to claim 12 wherein said ribozyme is produced *in vitro*.

15. The method according to claim 12 wherein said ribozyme is produced *in vivo*.

16. A method of interfering with or preventing hepatitis C virus (HCV) replication or gene expression in a cell infected with or susceptible HCV, which comprises introducing into the cell an effective amount of the ribozyme according to any one of claims 1 to 3.

17. A method of interfering with or preventing hepatitis C virus (HCV) replication or gene expression in a cell infected with or susceptible to HCV, which comprises introducing into the cell an effective amount of a vector according to claim 7.

18. The method according to claim 16 or 17 wherein said cell is a human cell.

19. The method according to claim 17 wherein said vector is a recombinant adenoviral or retroviral vector.

20. A pharmaceutical composition, comprising a ribozyme according to any one of claims 1 to 3, in combination with a pharmaceutically acceptable carrier or diluent.

21. A pharmaceutical composition, comprising a nucleic acid molecule according to claim 4 or 5, in combination with a pharmaceutically acceptable carrier or diluent.

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22. A pharmaceutical composition, comprising a vector according to any one of claims 7 to 10, in combination with a pharmaceutically acceptable carrier or diluent.

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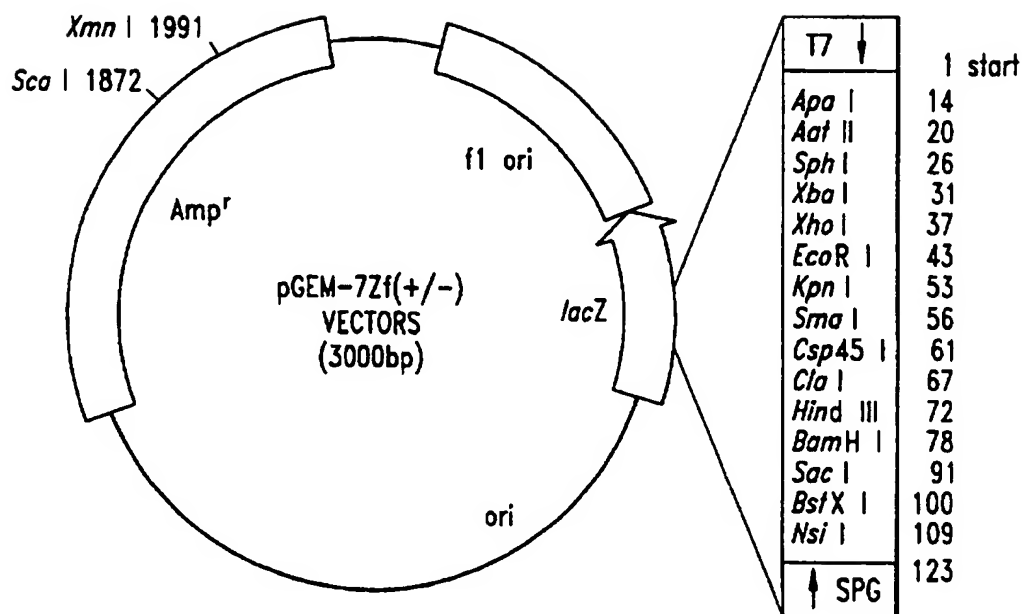


Fig. 1

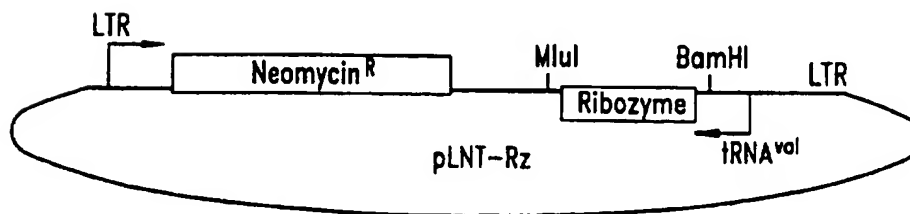
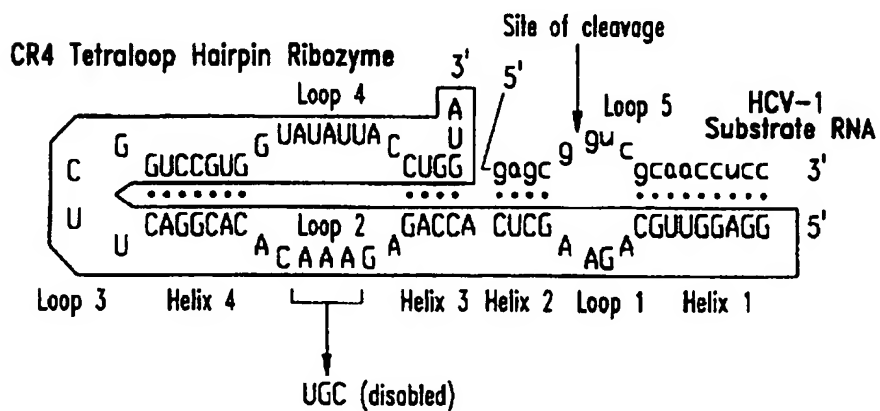
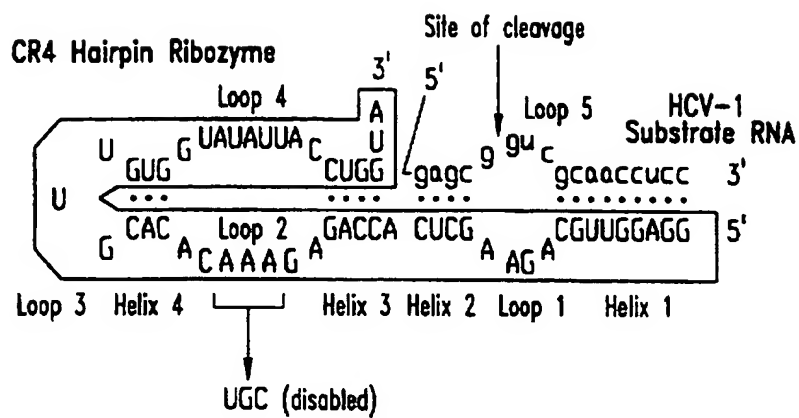
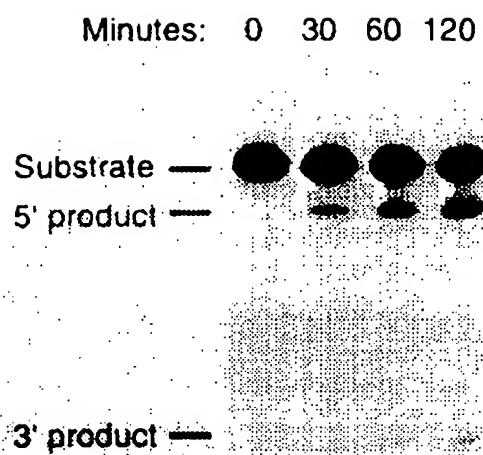


Fig. 2

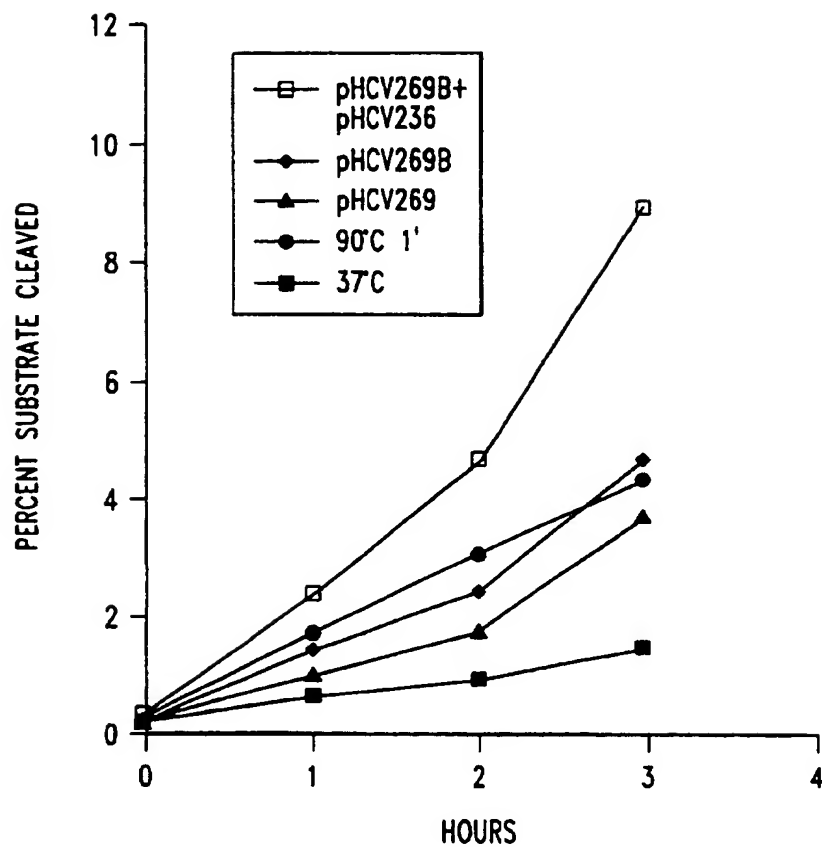
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*Fig. 3*

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*Fig. 4*

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*Fig. 5*

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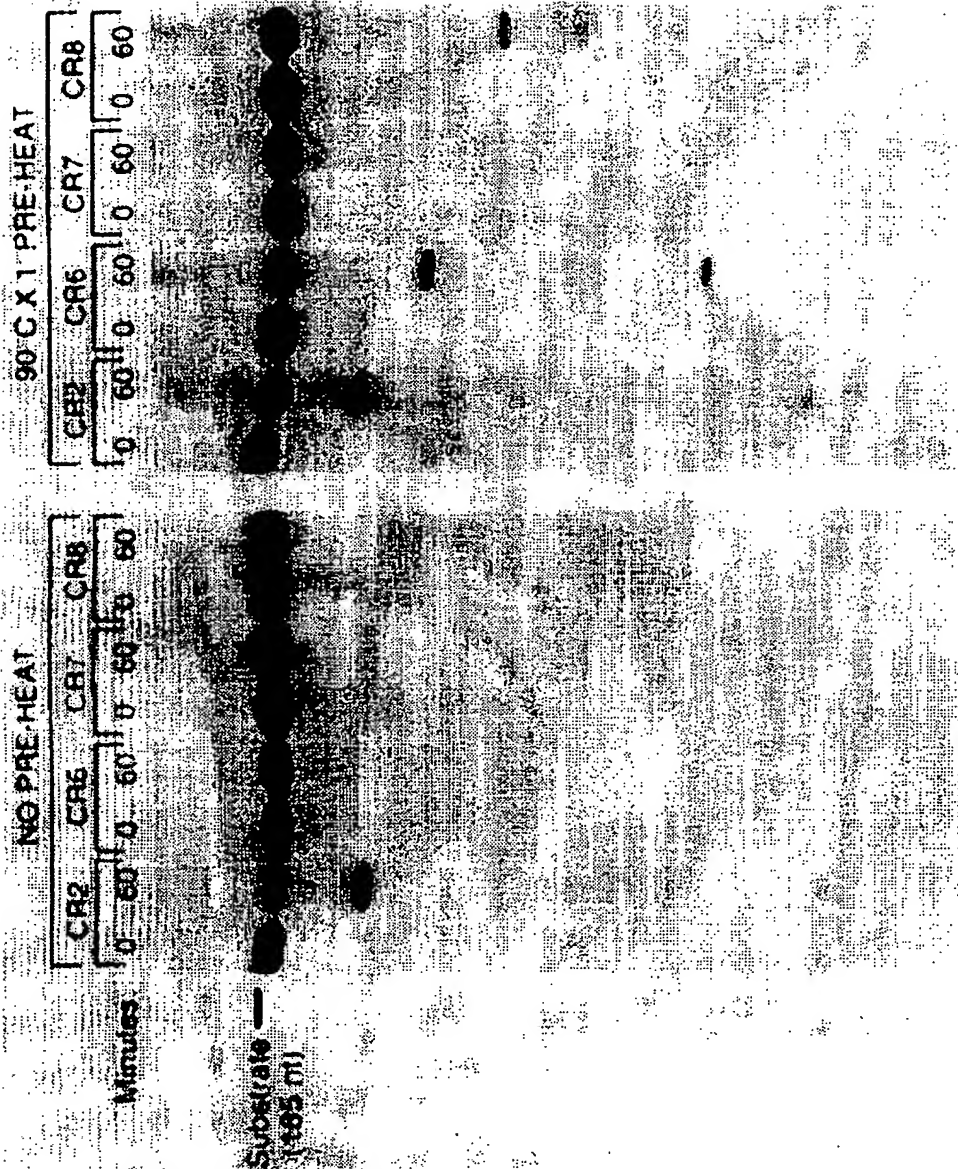
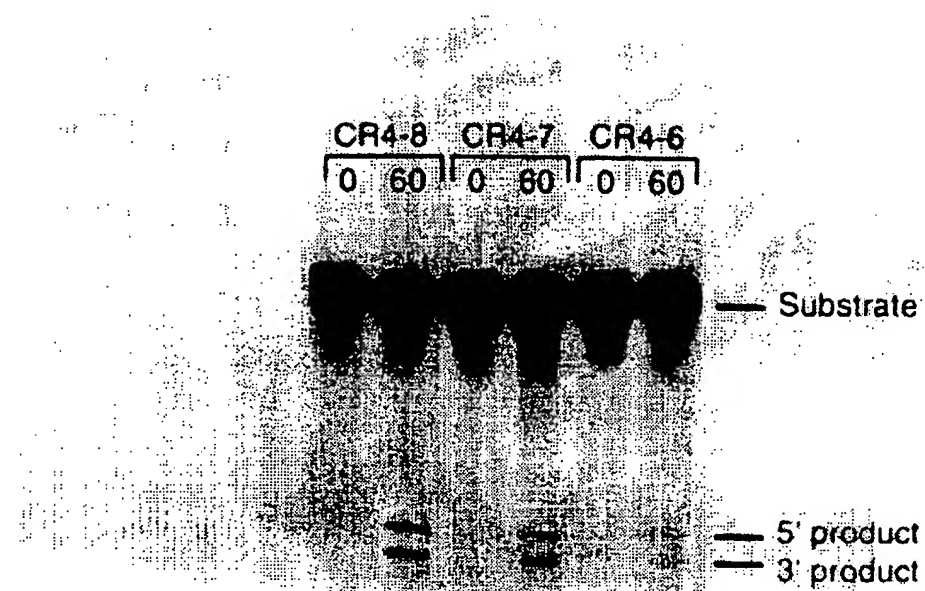


Fig. 6

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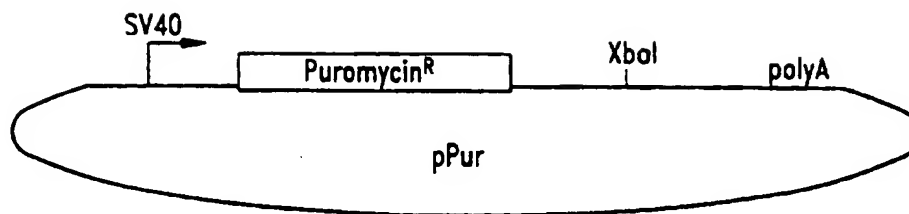
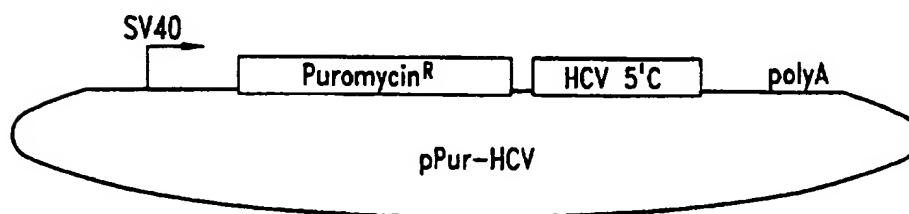
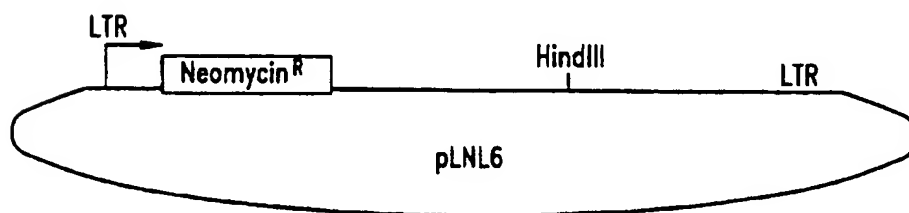
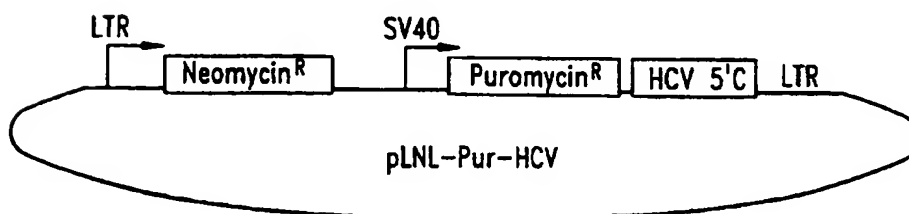
*Fig. 7*

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*Fig. 8*

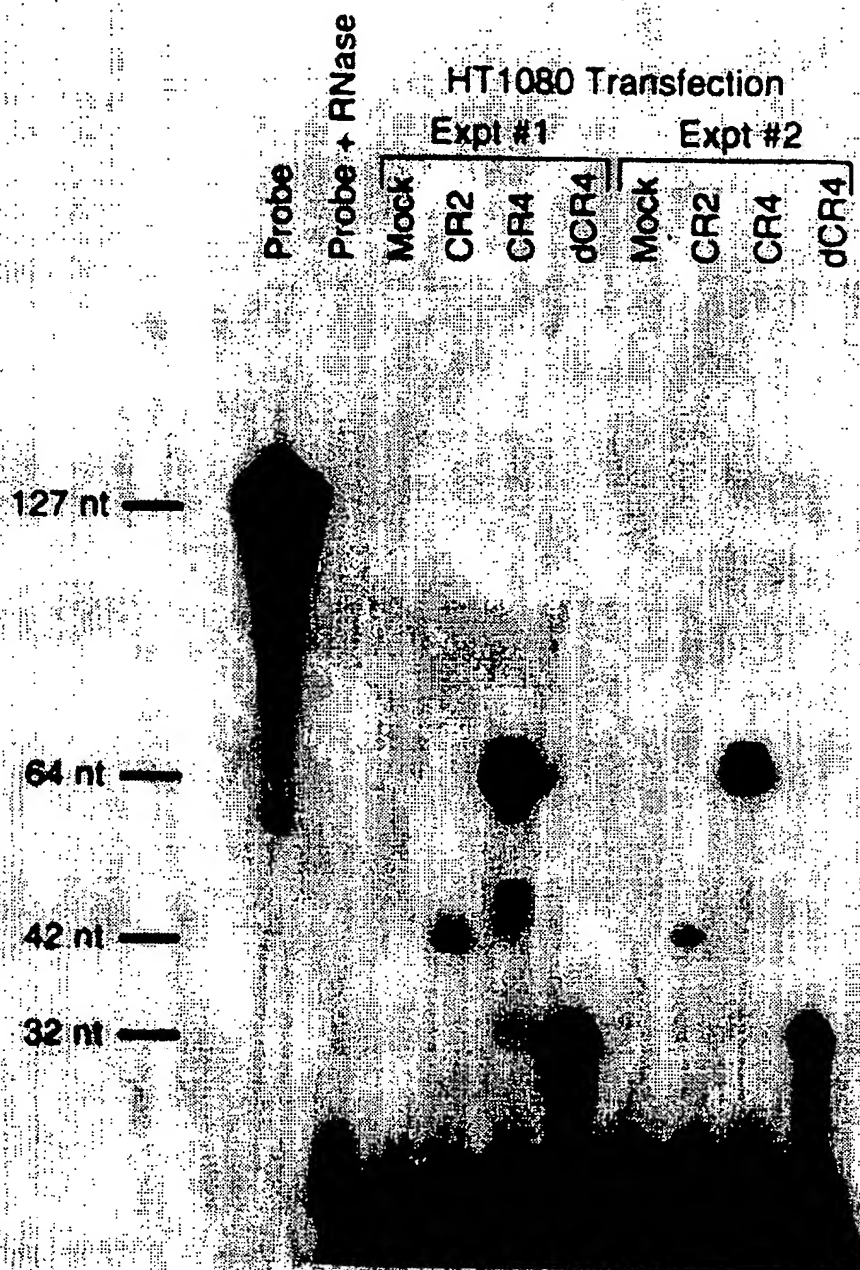
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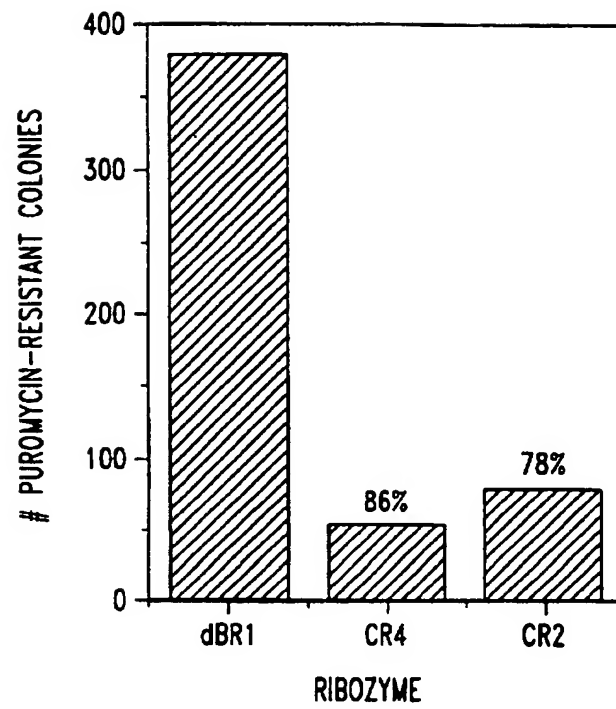
*Fig. 9A**Fig. 9B**Fig. 9C**Fig. 9D*

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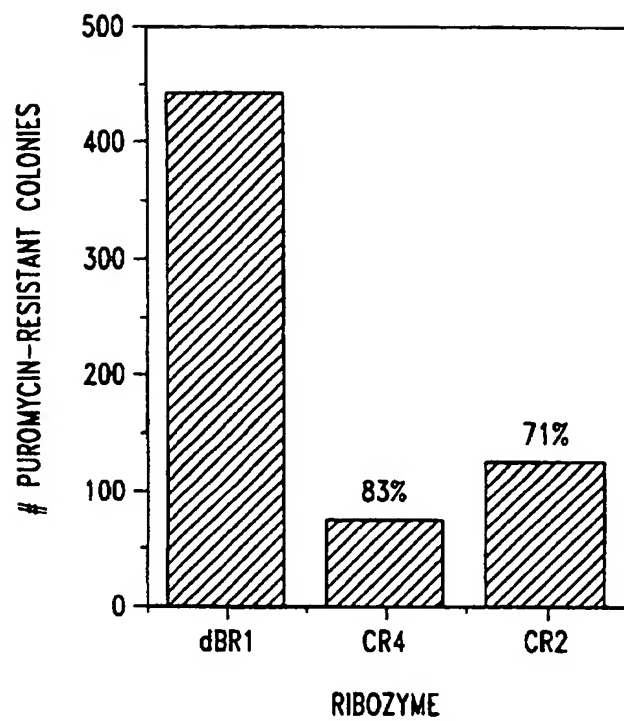
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*Fig. 10*

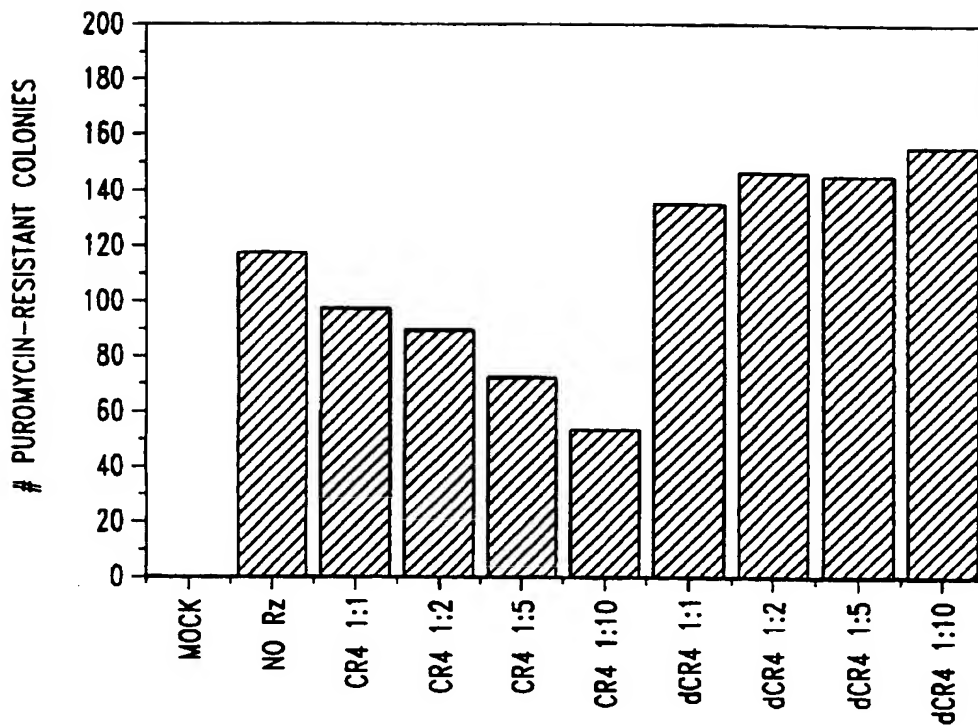
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*Fig. 11A*

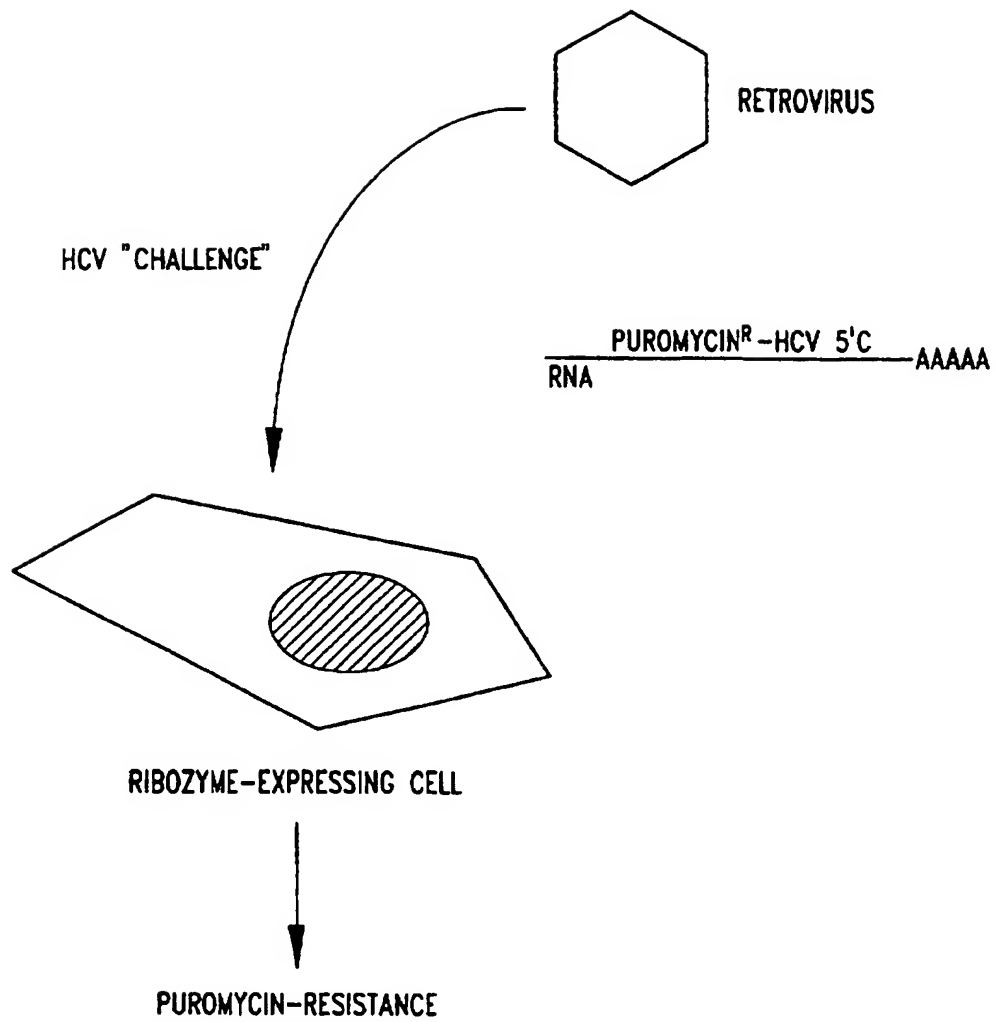
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*Fig. 11B*

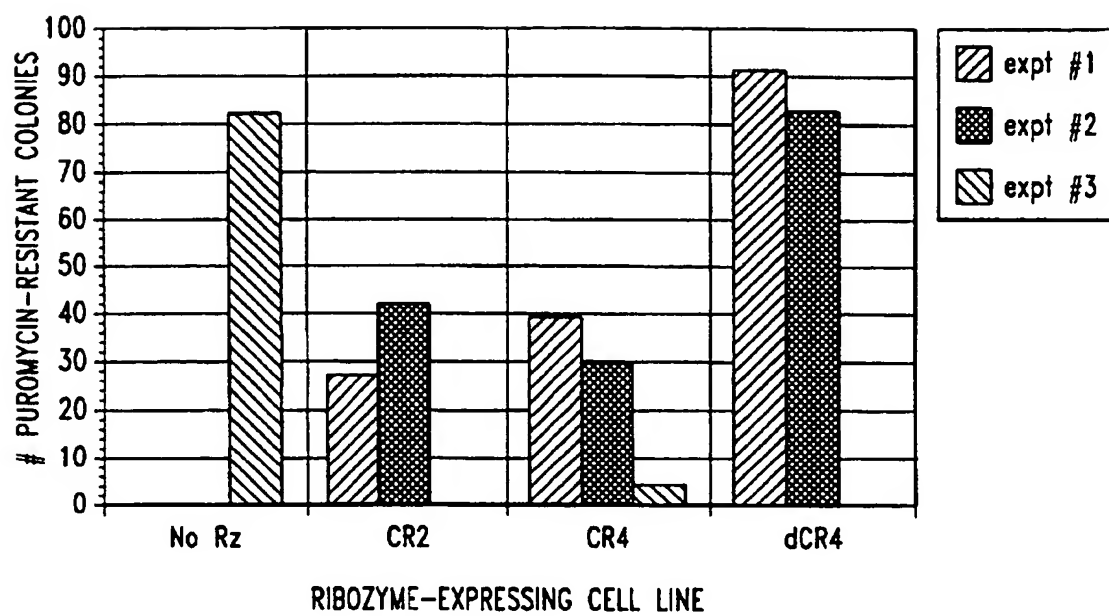
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*Fig. 11C*

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*Fig. 12*

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*Fig. 13*

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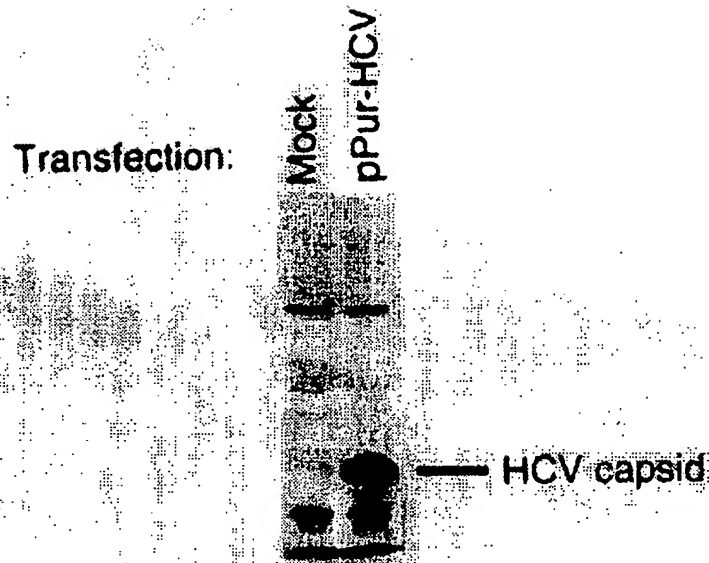


Fig. 14

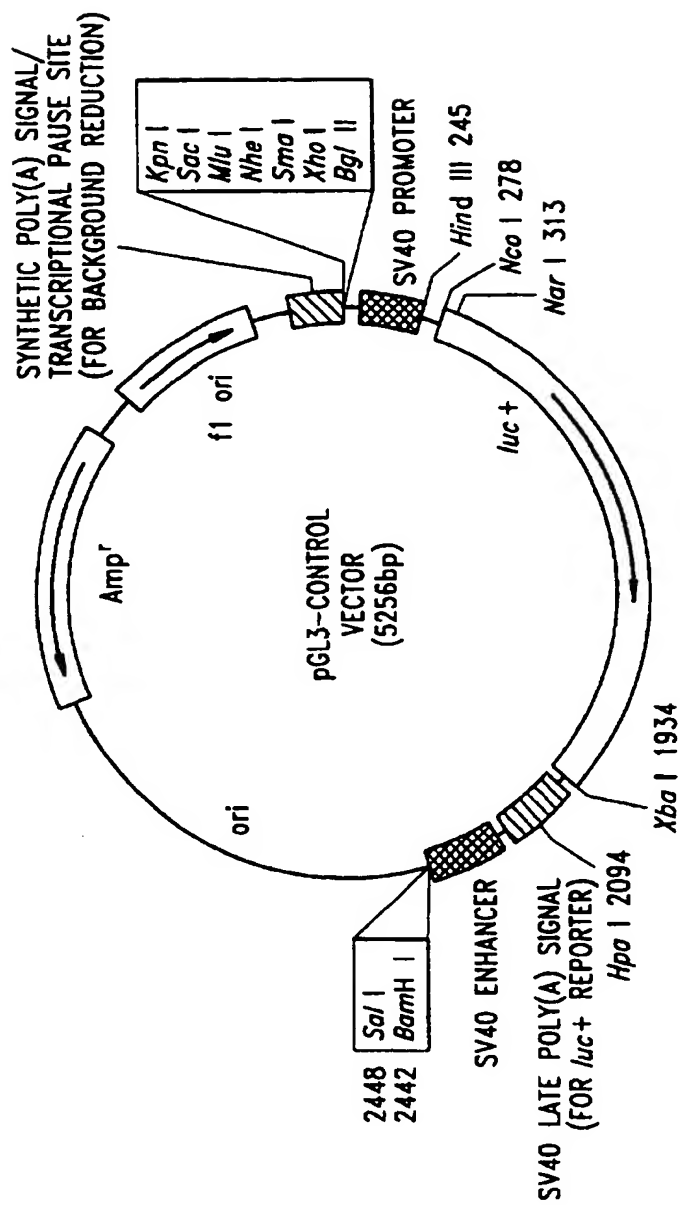


Fig. 15

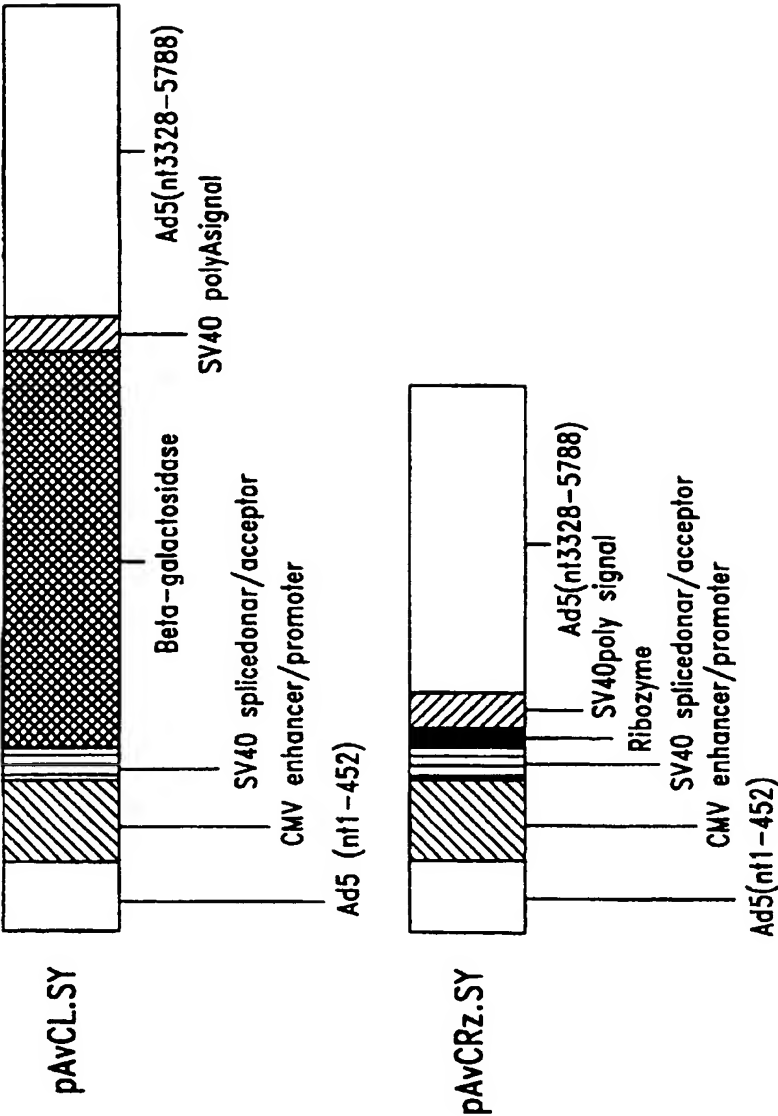
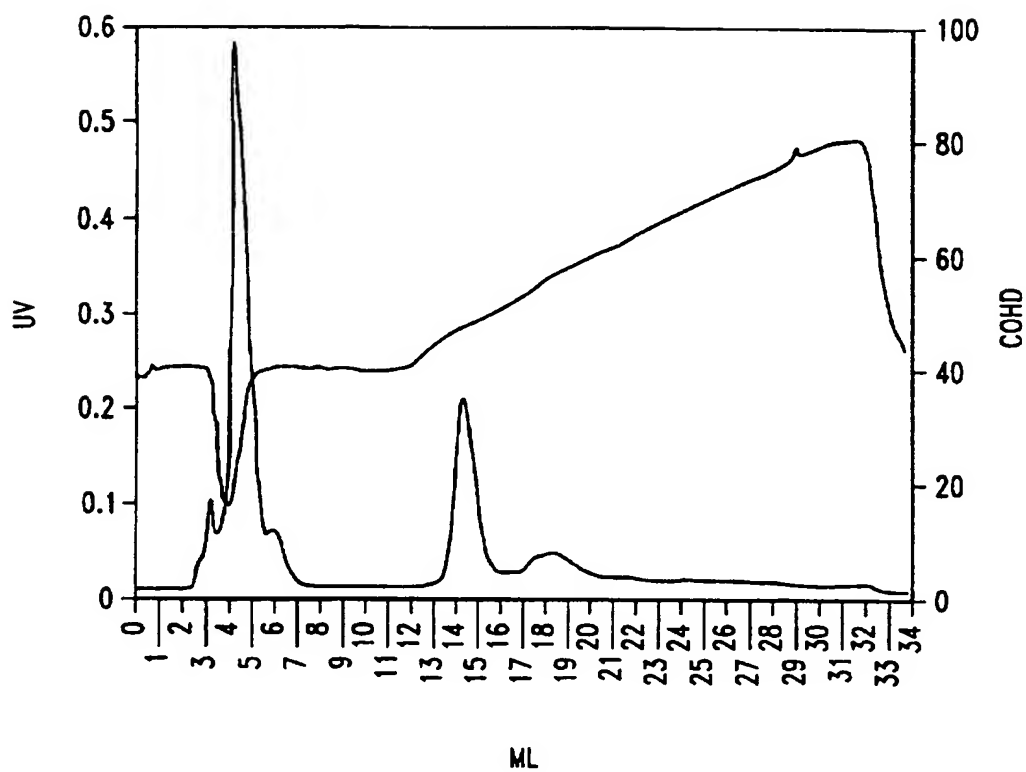
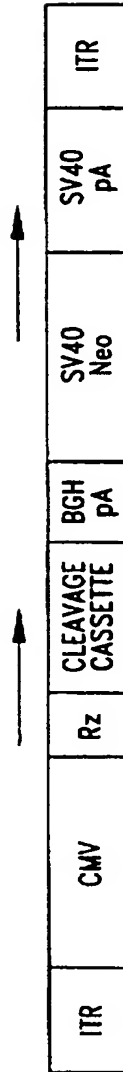


Fig. 16

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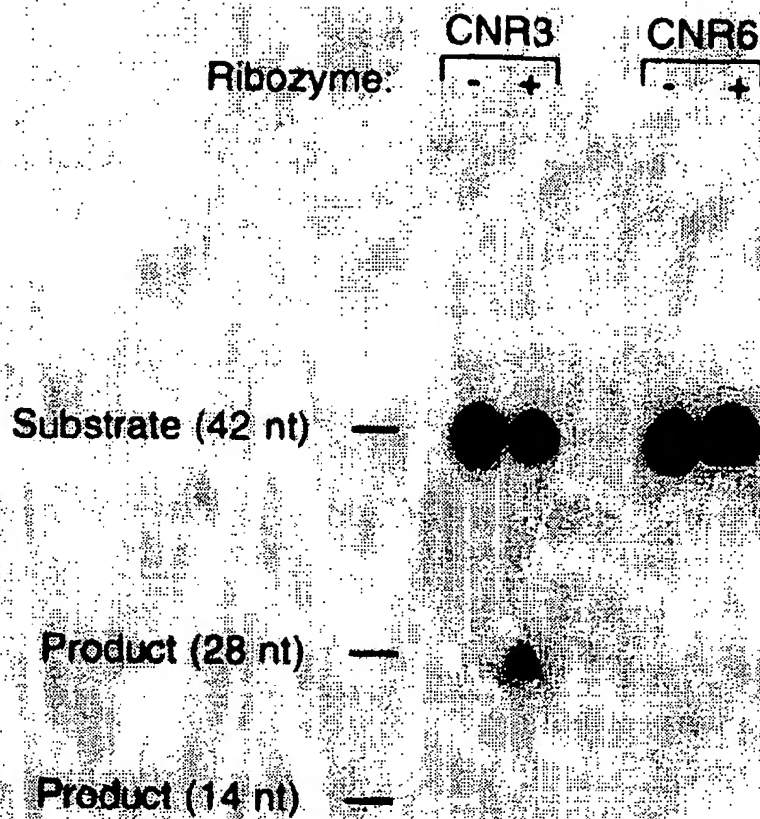
*Fig. 17*

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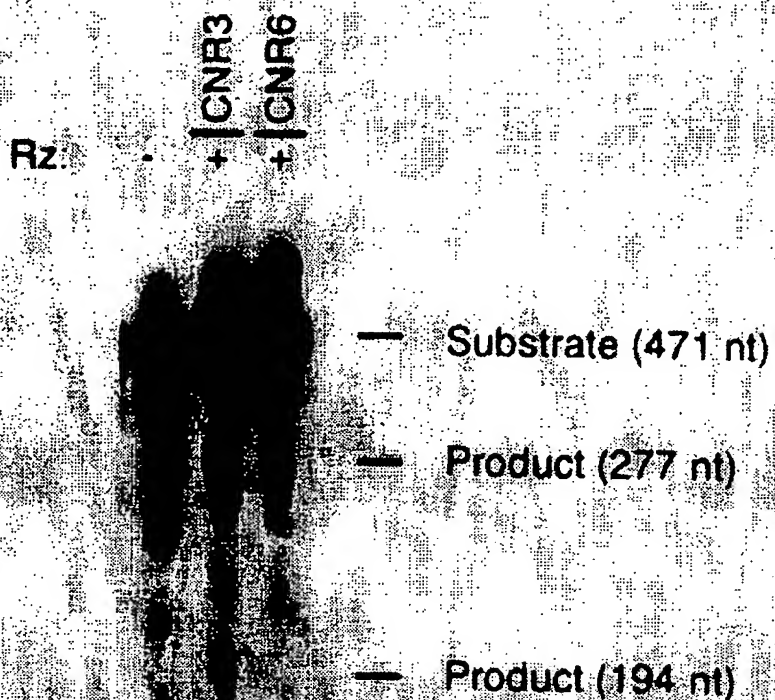
*Fig. 18*

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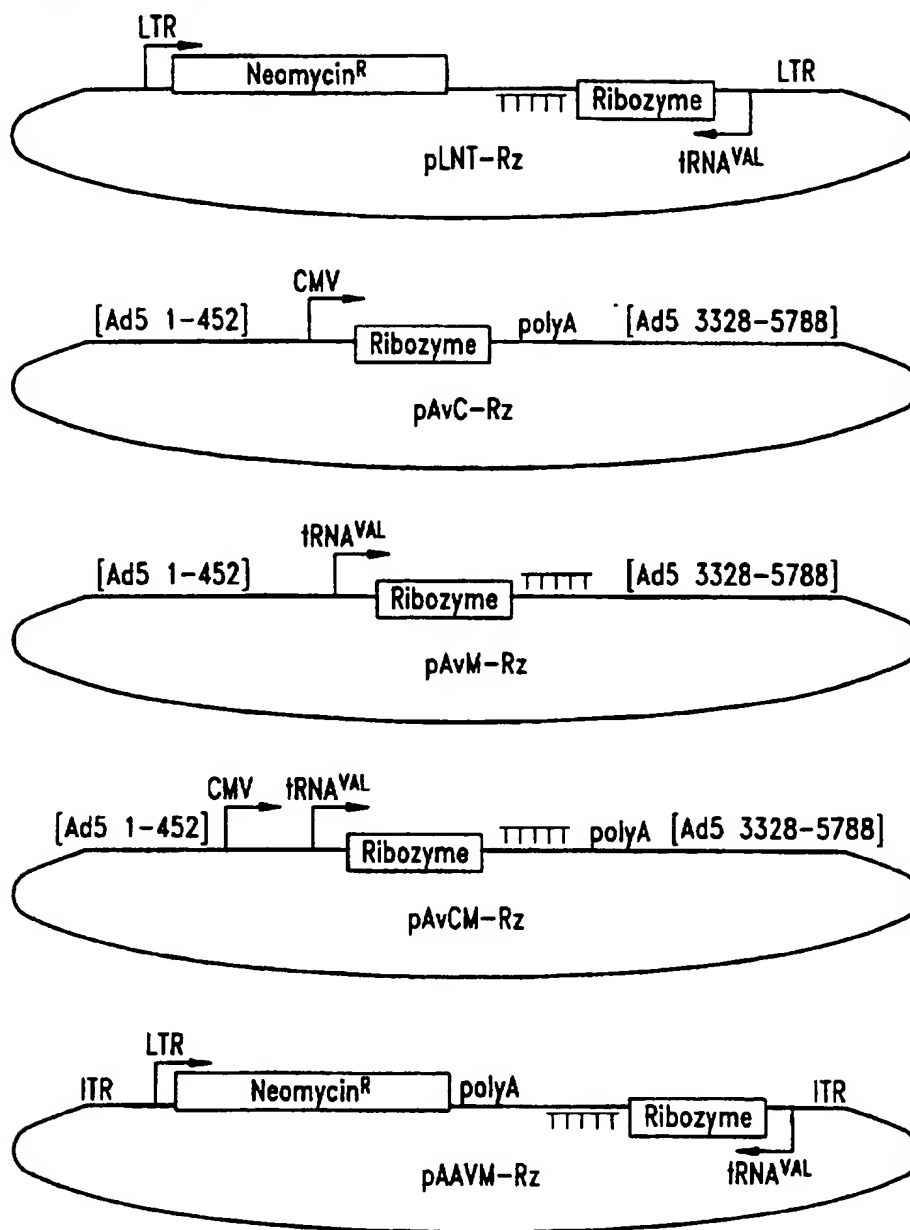
*Fig. 19*

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*Fig. 20*

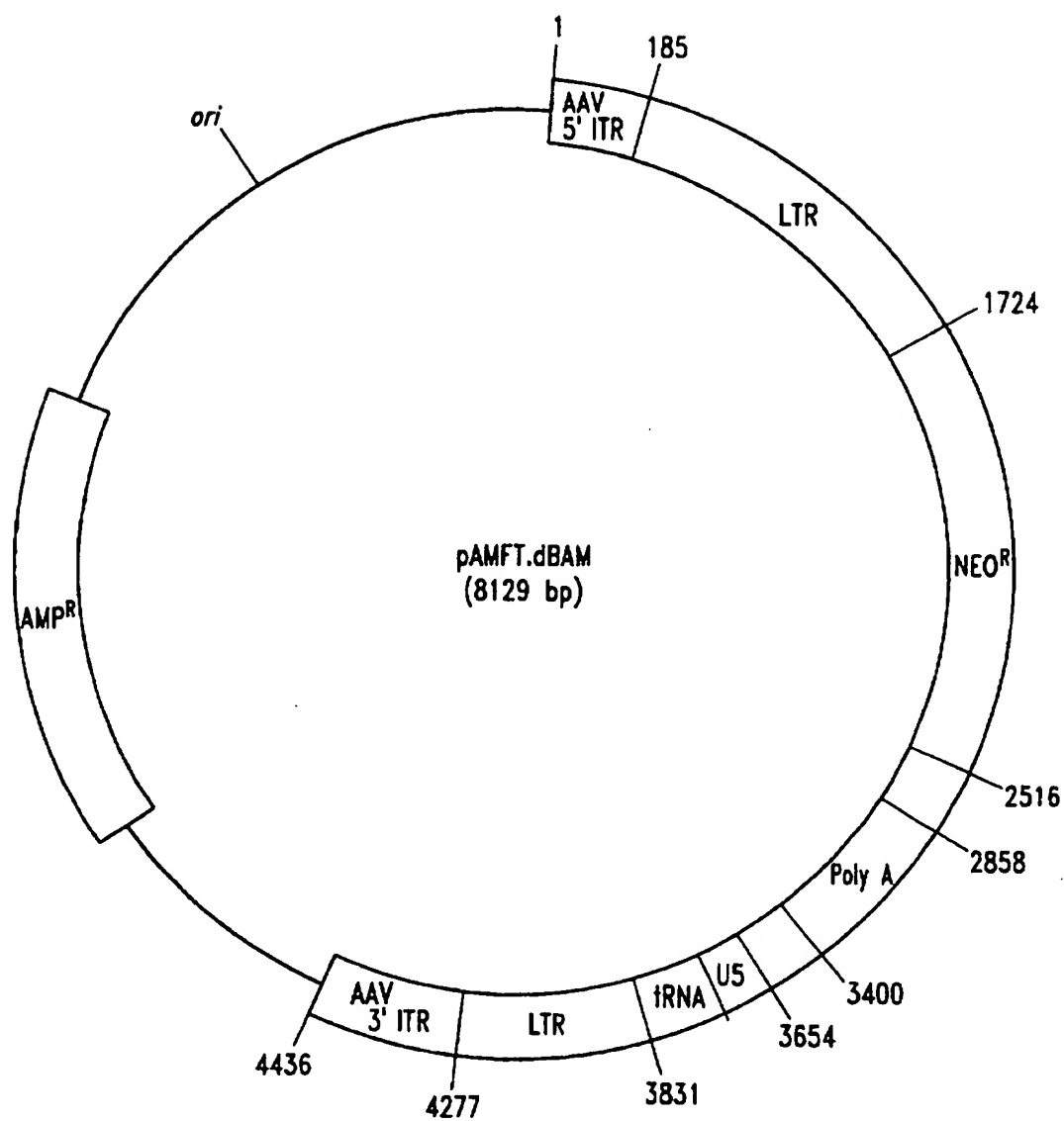
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Ribozyme expression:*Fig. 21*

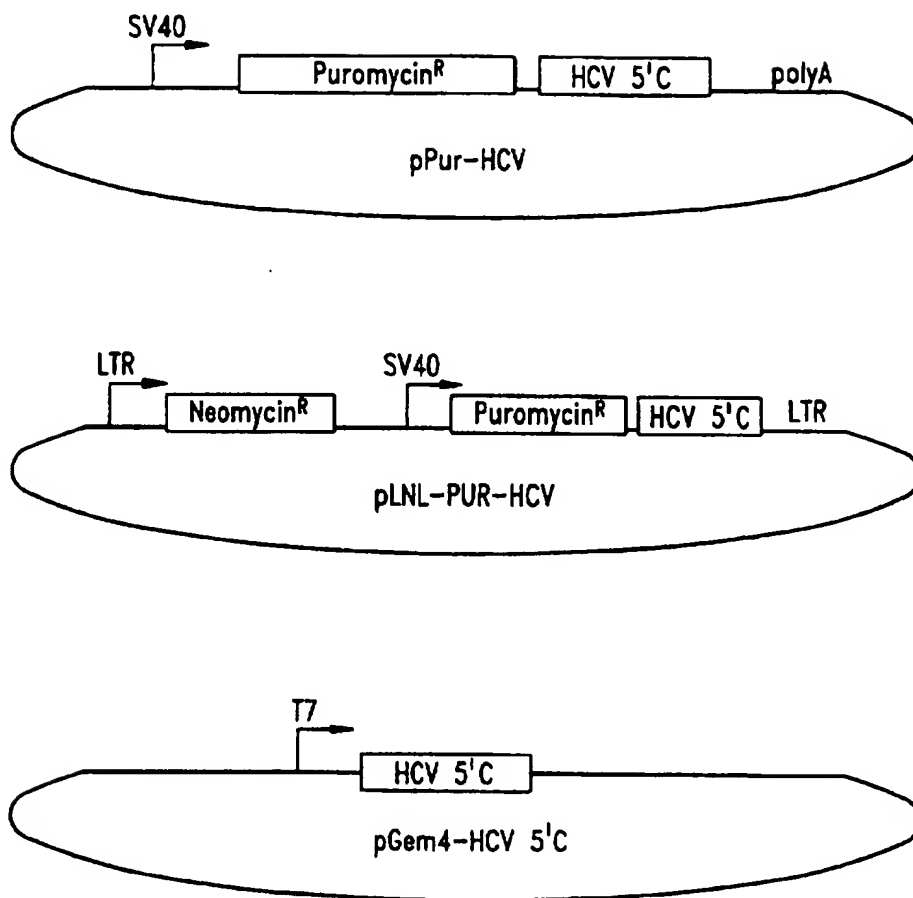
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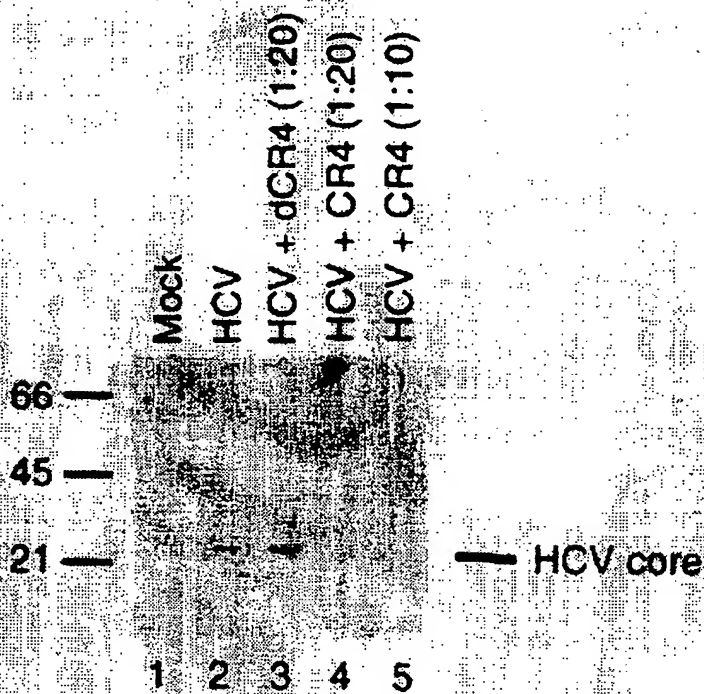
*Fig. 22*

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HCV expression:*Fig. 23*

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*Fig. 24*

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A. Promoter/Vector combinations

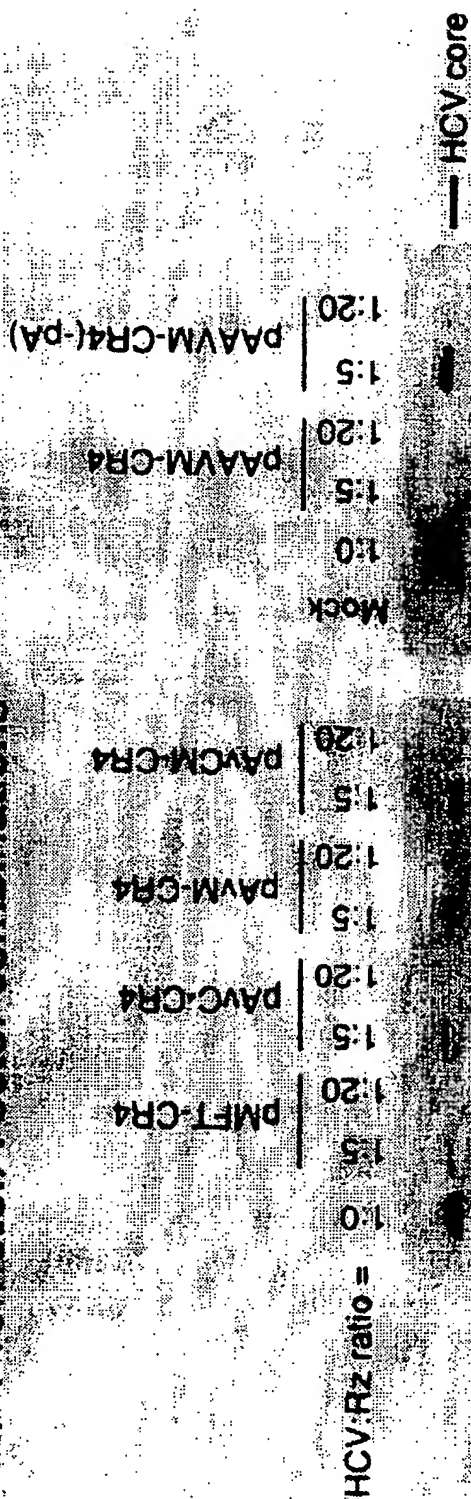


Fig. 25A

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B. Titration of pAAVM-CR4



Fig. 25B

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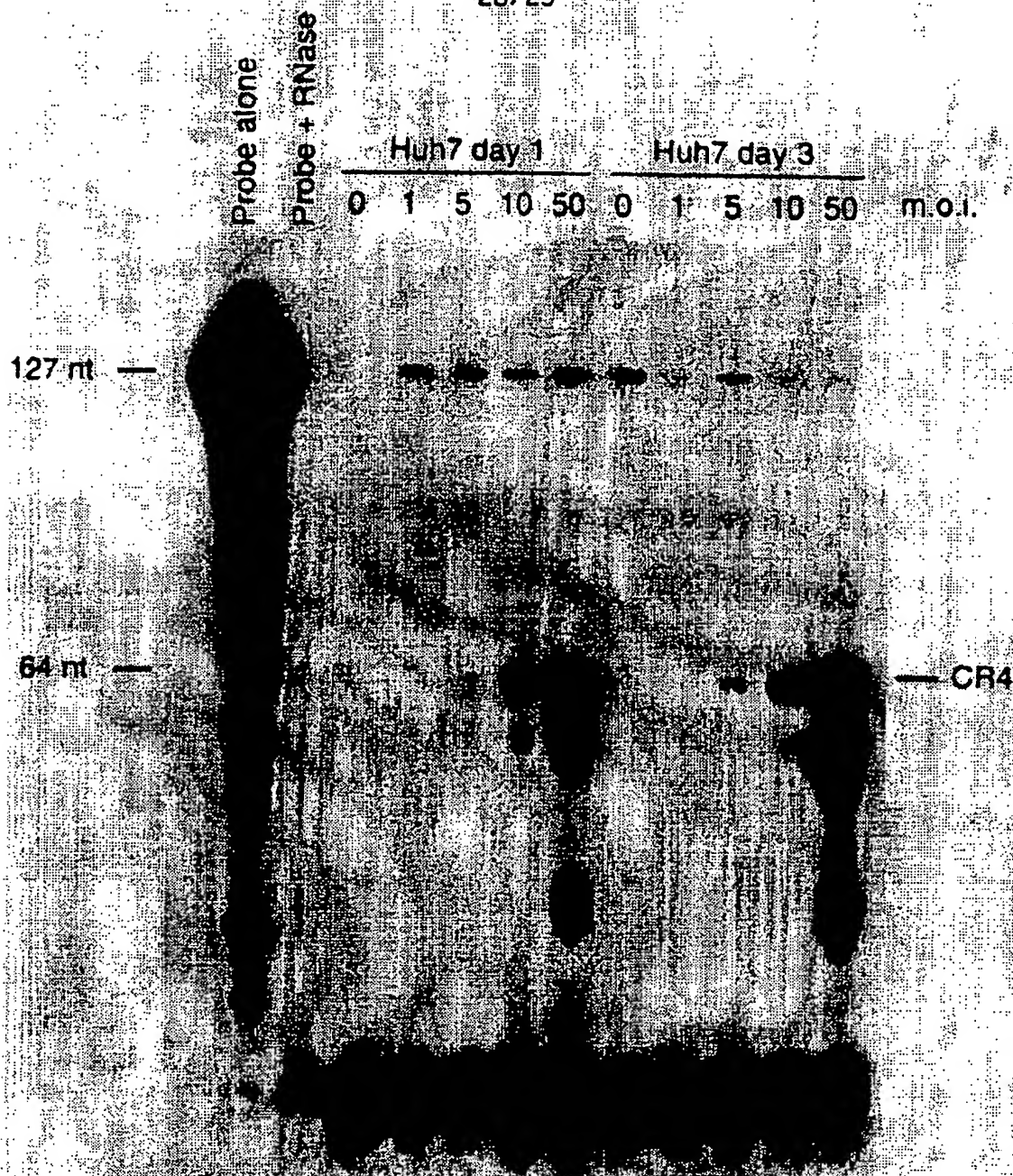
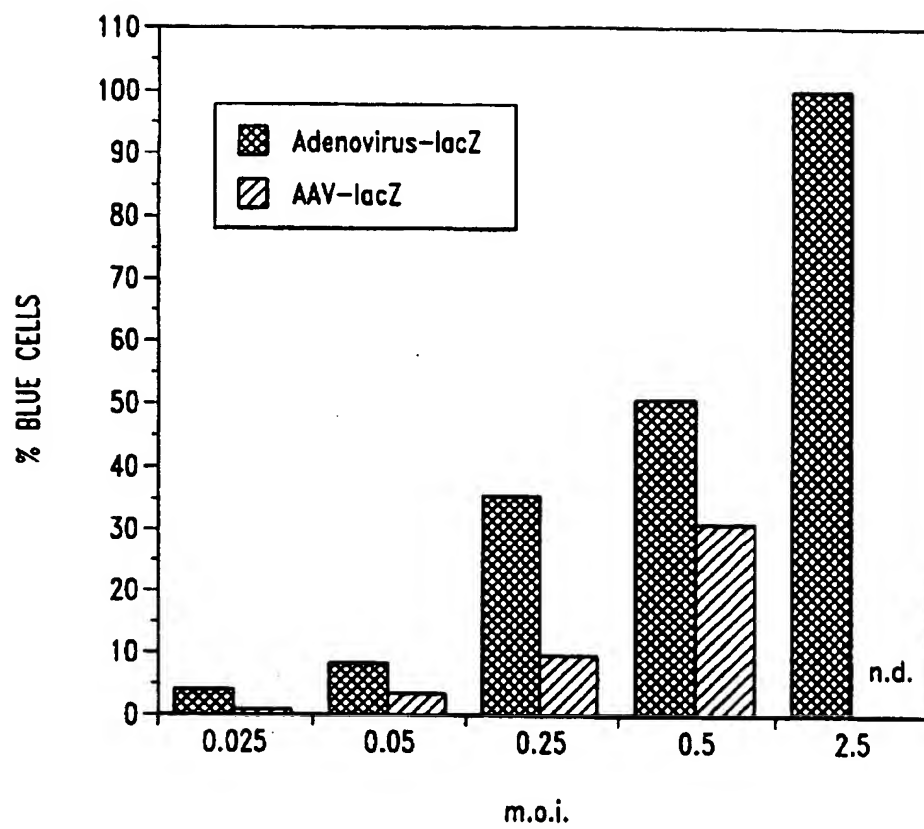


Fig. 26

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*Fig. 27*



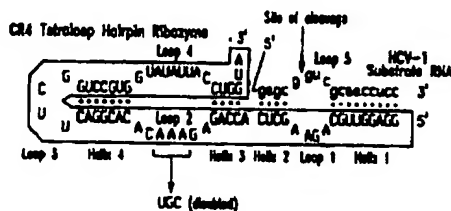
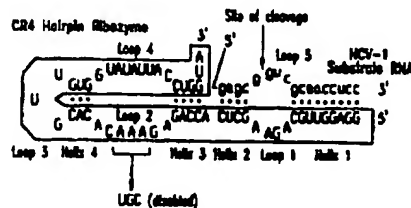
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(71) Applicant (for all designated States except US):	IMMUSOL, INC. [US/US]; 3050 Science Park Road, San Diego, CA 92121 (US).		
(72) Inventors; and			
(75) Inventors/Applicants (for US only):	BARBER, Jack, R. [US/US]; 11987 Cameridge Place, San Diego, CA 92128 (US). WELCH, Peter, J. [US/US]; 4689 East Talmadge Drive, San Diego, CA 92116 (US). TRITZ, Richard [US/US]; 9515 Genesee Avenue #127, San Diego, CA 92121 (US). YEL, SoonPei [US/US]; 7707 Sitro Musica, Carlsbad, CA 92009 (US). YU, Mang [CN/US]; 7155 B Calabria Court, San Diego, CA 92122 (US).		
(74) Agents:	McMASTERS, David, D. et al.; Seed and Berry L.L.P., 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).		

(54) Title: HEPATITIS C VIRUS RIBOZYMES

(57) Abstract

This invention provides ribozymes useful to treat or prevent Hepatitis C Virus ("HCV") infection or disease in an organism or subject, as well as methods of treating an HCV infection or disease. Reagents such as vectors, host cells, DNA molecules coding for these ribozymes useful in methods of treatment and prevention of HCV infection or disease are also provided.



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/03304

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/11 C12N15/52 C12N9/00 C12N15/86 A61K31/70 C12N5/10 //A61K48/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A61K C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 29241 A (CHIRON CORP) 2 November 1995 cited in the application see page 7, line 27 - page 8, line 13; figure 2 see page 17, line 12 - line 23 ---	1-22
X	WO 93 23569 A (RIBOZYME PHARMACEUTICALS INC) 25 November 1993 see page 53; example 7 see figure 12 see page 78, line 35 - page 89 see page 114, line 11 - line 26 see page 136; example 28 see page 182, line 31 - page 191 see claims 1,2,4,36-38,62 --- <div style="text-align: center;">-/-</div>	1-22
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
* Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*&* document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center;">7 August 1997</div>		Date of mailing of the international search report <div style="text-align: center;">21.08.97</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer <div style="text-align: center;">Andres, S</div>

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/03304

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 95 19429 A (RIBOZYME PHARM INC) 20 July 1995</p> <p>see page 5, line 23 - page 8 see page 36, line 10 - page 37, line 9 see page 47, line 4 - page 52 see claims</p> <p>---</p>	<p>1-9, 11-18, 20-22</p>
X	<p>EP 0 558 944 A (MAX PLANCK GESELLSCHAFT ;BAYER AG (DE); HOECHST AG (DE)) 8 September 1993</p> <p>see page 10, line 25 - line 48 see figure 7</p> <p>---</p>	<p>1,2,4,6, 7,9, 11-18, 20-22</p>
A	<p>NUCLEIC ACIDS RESEARCH, vol. 22, no. 6, 25 March 1994, pages 1096-1100, XP000567885 ANDERSON P ET AL: "MUTAGENESIS OF THE HAIRPIN RIBOZYME" cited in the application</p> <p>---</p>	
A	<p>ANTISENSE RESEARCH AND DEVELOPMENT, vol. 4, 1994, US, pages 243-249, XP002037123 NESBITT, S. & GOODCHILD, J.: "Further studies on the use of oligonucleotide facilitators to increase ribozyme turnover"</p> <p>---</p>	
X,P	<p>WO 96 18419 A (UNIV WASHINGTON ;KAY MARK A (US); LIEBER ANDRE (US)) 20 June 1996 see page 7, line 19 - page 8, line 16 see page 16, line 37 - page 26, line 26 see examples VI-VII, IX see figure 8</p> <p>---</p>	<p>1-22</p>
P,X	<p>GENE THERAPY, 3 (11) 994-1001, November 1996, XP000653315 WELCH, P. ET AL.: "A potential therapeutic application of hairpin ribozymes: in vitro and in vivo studies of gene therapy for hepatitis C virus infection." cited in the application see the whole document</p> <p>-----</p>	<p>1-22</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/03304

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: X
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 12, 16-19 (as far as in vivo methods are concerned) and claim 15 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 97/03304

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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